

Characterization of major capsid protein VP7 of African horsesickness virus as an antigen display system

by

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Declaration

I, Daria Anna Rutkowska, declare that the thesis, which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

Date:

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SUMMARY

Characterization of major capsid protein VP7 of African horsesickness virus as an antigen display system

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Due to the poor stability and immunogenicity of small antigenic peptides, it is necessary that they be presented to the immune system in conjugation with larger carrier proteins or on the surface of chimeric viral or virus-like particles in order to elicit an immune response. When the major capsid protein VP7 of African horsesickness virus (AHSV) is expressed in *Spodoptera frugiperda* (Sf9) insect cells by means of a recombinant baculovirus strategy, the VP7 trimers aggregate and are assembled into unique, distinctive crystal-like particles. Immunization of mice with AHSV-9 VP7 crystal-like particles has been found to protect 80-100% of the animals against virulent, heterologous AHSV-7 challenge (Wade-Evans et al., 1997). These results suggested the possibility of using such particles for the immune display of foreign antigenic peptides. Natural hydrophilic loops within the AHSV-9 VP7 proteins' top domain were targeted for the display of foreign peptides by inserting multiple cloning sites into three different positions within the gene encoding the VP7 protein. In this study we explored the feasibility of using the AHSV major capsid protein VP7 as an antigen display system by investigating how peptide insertions into the VP7 top domain affect VP7 particle formation, solubility, trimer formation and immune display.

To generate the VP7 vectors with multiple cloning sites, small 6 amino acid peptides were inserted downstream of amino acids 144, 177 and 200 within the VP7 top domain in previous studies. These VP7 vectors were

subsequently used for the insertion of a range of peptides that varied in size from 11 to 150 amino acids. These peptides were derived from outer capsid protein VP1 of foot-and-mouth-disease virus (FMDV), the major neutralization-specific antigen of AHSV (VP2) and well as the envelope protein gp41 of HIV-1. The largest sequence to be inserted into the VP7 top domain was the 239 amino acid enhanced green fluorescent protein (eGFP). To address the question of the effect of foreign peptide insertion on AHSV-9 VP7 particle formation and protein solubility, the expressed chimeric VP7 proteins were analyzed by sucrose density gradient sedimentation analysis. The effect of these peptide insertions on the ability of the AHSV-9 VP7 protein to form trimers was investigated by either a low density sucrose sedimentation strategy or a modified SDS-polyacrylamide gel electrophoresis strategy. The ability of an inserted peptide to elicit an immune response, when presented on the surface of the VP7 trimer, was determined by inoculating the chimeric VP7 fusion protein into guinea pigs and analyzing the humeral immune response elicited by means of insert-specific ELISA and neutralization assays. Since these insertions affected VP7 protein solubility to a significant degree, an important question that was addressed was whether the chimeric VP7 particles or the soluble chimeric VP7 trimers presented the best display strategy for eliciting a strong insert-specific humeral immune response. The effect of various solubilization strategies, such as protein sample dilution and treatments with the solubilization agents L-arginine and sarkosyl, on chimeric VP7 protein solubility were also investigated as well as the effect of these treatments on the immunogenicity of the inserted peptide.

The insertion of different 6 amino acid peptides downstream of amino acids 144, 177 and 200 in the AHSV-9 VP7 top domain during vector construction affected VP7 protein solubility. The inserted peptide and insertion site were both found to play a role. The insertion of a 6 amino acid peptide downstream of VP7 amino acid 144 converted the hydrophobic VP7 protein to a largely soluble protein whereas the insertion of such a peptide downstream of amino acid 200 had no apparent effect on solubility. Although the ability of the VP7 protein to form trimers was not affected, the stability of these trimers under strong denaturing conditions, such as in the presence of SDS at room temperature, was dependent on the site of insertion. Trimers with peptides inserted downstream of VP7 amino acid 177 were the most stable whereas the insertion of peptides downstream of amino acid 200 resulted in the least stable trimer molecules. The insertion of larger foreign peptides into the VP7 vector proteins also influenced protein solubility. Insertion of a 36 amino acid peptide from the FMDV VP1 protein (aa129-164) into site 177 yielded the largely soluble chimeric VP7-177(P₁)-FMDVepi protein. A marked reduction in solubility was observed when the same peptide was inserted into a different site. VP7 solubility and particle formation was also strongly affected by the length and amino acid composition of the inserted peptide. This demonstrated that both the VP7 top domain insertion site and the nature of the inserted peptide influence the overall equilibrium between the soluble VP7 chimeric trimers and VP7 particles. It is, as yet, not possible to predict chimeric VP7 protein solubility and the solubility of each construct needs to be individually assessed. The insertion of these larger peptides did not affect the ability of the VP7 fusion proteins to form trimers but trimer stability was again affected as described in the case of the VP7 vector proteins. The only exception was the VP7-200(P₁)-eGFP protein, the majority of which occurred in a monomeric rather than in a trimeric form. As both

the VP7-144(P₂)-eGFP and VP7-177(P₁)-eGFP fusion proteins formed trimers, this indicated that the AHSV VP7 trimer is a stable molecule capable of accommodating even large insertions within its top domain without losing its ability to form trimers.

A comparison of the insert-specific humeral immune response elicited against the FMDV VP1 peptide (aa129-164) presented either on VP7-FMDVepi particles or soluble VP7-FMDVepi trimers indicated that, whereas the particles elicited a weak FMDV-specific, neutralizing humeral immune response in guinea pigs, the soluble trimers induced a much stronger immune response. This result focused much of our investigation away from the use of insoluble AHSV VP7 particles as an antigen display system to the use of soluble AHSV VP7 trimer molecules. The large quantity (6.5mg) of soluble VP7-177(P₁)-FMDVepi trimers harvested from 1×10^8 baculovirus-infected insect cells made this a feasible strategy. In order to increase the soluble component of less soluble VP7 proteins, various strategies were investigated. One such strategy involved the substitution of an uncharged leucine residue in position 345 within the bottom domain of the AHSV VP7 protein, with a positively charged arginine residue, the bluetongue virus (BTV) VP7 equivalent. Not only did this mutation significantly increase the solubility of the modified VP7 proteins, their ability to form stable trimers was not affected. This modification may thus be incorporated into future vector proteins to increase VP7 protein solubility. Treatments aimed at reversing VP7 trimer-trimer aggregation were also investigated as a means of increasing VP7 protein solubility. Dilution of the protein sample and/or treatment with the amino acid L-arginine (Tsumoto et al., 2004) was found to reverse the loose aggregation of correctly folded VP7 fusion trimer molecules while treatment with the ionic detergent Na⁺N-Lauroylsarcosine (Sarkosyl) solubilized both correctly folded and incorrectly folded trimer aggregates. Inoculation of the chimeric VP7 antigen in the presence of L-arginine or sarkosyl elicited a more consistent, strong immune response than untreated, soluble VP7-177(P₁)-FMDVepi trimers. This indicated the importance of presenting soluble, non-aggregated antigen to the humeral immune system and that these treatments did not negatively affect antigen immunogenicity. Although both the L-arginine and sarkosyl treatments elicited equivalent humeral immune responses against the VP7-177(P₁)-FMDVepi trimers, L-arginine is not required to be removed from the immunogen solution prior to inoculation and, as this amino acid is capable of solubilizing aggregates of correctly folded trimers, it is currently the preferred solubilization protocol.

The eGFP protein folds equally well into its functioning, fluorescing conformation when inserted into any of the three top domain sites. Thus peptides inserted into these sites would most likely have the opportunity to fold correctly into their native conformations. It was, however, observed that the chimeric VP7-144-eGFP fusion protein, although apparently correctly folded and fluorescing, had a tendency to form loosely assembled aggregates that were readily solubilized by simple dilution or treatment with L-arginine. This was in contrast to the VP7 fusions with eGFP inserted at sites 177 and 200, which both had smaller soluble fluorescing fractions, and were not significantly affected by these solubilization treatments. The tendency of correctly folded VP7-144-eGFP fusion trimers to assemble into loosely aggregated particles is also observed with other inserted peptides,

such as the AHSV VP2 110 amino acid peptide, and has formed the basis of a low speed centrifugation strategy designed to harvest large quantities of correctly folded, soluble VP7 fusion trimers.

The VP7-HIV fusion proteins were found to be largely insoluble when expressed in insect cells via a recombinant baculovirus strategy and were subsequently explored with respect to their potential to detect HIV antibodies in HIV patient sera. The different binding profiles observed on immune blots against a range of VP7-HIVgp41 fusion proteins could be used to characterize HIV-1 antisera as either subtype B or C but this strategy needs to be further explored. In one of the sera an antibody response against the small ELDKWA epitope on the gp41 protein of HIV-1 subtype B was detected.

The results of this study indicate that foreign peptides may be displayed by AHSV major capsid protein VP7 either on the surface of soluble VP7 trimers as a vaccine display system or by insoluble VP7 particles, in a denatured form on immunoblots, as an antiserum characterization strategy. As it is possible to produce more soluble AHSV VP7 trimers by modifying the VP7 protein, it may also be possible to increase the yield of core-like particles (CLPs) when this protein is co-expressed with AHSV VP3 in recombinant baculovirus-infected cells. Although the size of the presented peptide may be limited by a CLP strategy, these larger particles may induce cellular immunity. Although not yet investigated, it is likely that the soluble VP7 trimers may only elicit a humeral and not a cellular immune response.

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Chapter 1

Literature review

1. 1 Introduction

The science of vaccinology is considered to have its origin in the late 18th century when Edward Jenner administrated cowpox as a vaccine against smallpox (Plotkin & Plotkin, 2004). This action was based on Jenner's observation that milkmaids who suffered from the less pathogenic cowpox infection were typically protected against the pathogenic smallpox disease. Thus the paradigm was established that the administration of a relatively non toxic or nonpathogenic counterpart of the pathogenic agent or organism could result in a pathogen-resistant (immune) state with minimal risk to the individual (Germain, 2010).

Traditionally, vaccines have consisted either of inactivated or live attenuated pathogenic microorganisms (Plotkin, 2009). Inactivated vaccines use a killed form of the microorganism responsible for the disease that is inactivated following treatment with chemical or physical agents (heat or radiation). Inactivated vaccines include those currently available against hepatitis A, influenza, foot-and-mouth disease (FMD) and bluetongue (BT) disease (Nichol, 2003, Paton & Taylor, 2011, Savini et al., 2008). The concerns associated with the use of these vaccines include the risk of incomplete inactivation of the pathogen and the large antigen dose required to induce an immune response. Live attenuated vaccines, such as those currently used against measles, rubella, mumps, yellow fever, bluetongue and African horsesickness (AHS) are obtained via serial passage of the pathogens in tissue culture resulting in the loss or mutation of genes involved in pathogenicity, virulence and immune evasion (Mortellaro & Ricciardi-Castagnoli, 2011, Savini et al., 2008, von Teichman & Smit, 2008). Attenuated vaccines have been responsible for reducing the occurrence of polio to a few small pockets worldwide and completely eliminating the smallpox disease (Plotkin, 2009). Although successful, the use of attenuated vaccines is associated with a risk of reversion to virulence and there are safety concerns regarding their use in immunocompromised individuals (Batten et al., 2008, Ehrenfeld et al., 2009, Savini et al., 2010).

The reverse genetics technology has allowed for the introduction of defined mutations into viral genomes resulting in the production of rationally attenuated vaccines (Boyce et al., 2008, Castrucci et al., 1992, Halfmann et al., 2009). Replication-defective bluetongue viruses, synthesized using this technology in combination with a complementing VP6 cell line, have recently been shown to elicit neutralizing antibodies that protected the host animals from virulent viral challenge (Matsuo et al., 2011). The advantage of this strategy is that, due to deletion mutations within a replication-essential gene (Matsuo & Roy, 2009), the defective virus strains are only able to go through a single cycle of replication within the target host cell. The lack of replication of these defective viruses in the host animals would make them less likely to revert to a virulent strain, a concern with typical attenuated viruses, thus making them as safe as inactivated vaccines while at the same time preserving the

expression of viral proteins at the natural sites of infection, as with live vaccines. Another advantage of this strategy is that it makes it possible to differentiate between infected and vaccinated animals. The absence of antibodies against VP6 was proposed as a marker for vaccinated and non-infected animals. The use of the so-called Differentiation of Infected from Vaccinated Animals or 'DIVA' vaccines in areas where a disease is not endemic allow for vaccination whilst still enabling the possibility of serological surveillance for the presence of infection (Uttenthal et al., 2010). These vaccines would also enable the export of vaccinated, non-infected livestock from endemic countries. This is particularly important in the thoroughbred horse industry in South Africa where a outbreak of African horsesickness disease in a control area can result in a ban of exports for at least 2 years (Sinclair et al., 2006).

Other DIVA-compliant alternatives to the current inactivated and live attenuated vaccines include recombinant protein subunit vaccines, peptide based vaccines, DNA vaccines, live recombinant viral vectors and virus-like particles (VLPs). Recombinant protein subunit vaccines direct the immune response towards immunologically relevant proteins from pathogenic agents (Plotkin, 2009) while peptide-based vaccines present one or a few epitopes to the immune system on a synthetic peptide (Brun et al., 2011). DNA vaccines have been used to deliver DNA encoding immunogenic proteins or peptides into target cells (Arrode-Bruses et al., 2010) as have recombinant viral vectors (McConkey et al., 2003). The presentation of immunogens on the surface of virus-like particles, which lack the viral genetic component, have been found to induce both strong cellular and humeral immune responses (Jegerlehner et al., 2002, Jennings & Bachmann, 2007, Mizukoshi et al., 2009). Immunologically important proteins and peptides have also been presented on the surface of particulate viral structures, such as the P particle of Norovirus (Tan et al., 2011) or the NS1 tubules of Bluetongue virus (Murphy & Roy, 2008). The African horsesickness viral protein 7 has been shown to assemble into crystal-like particles when expressed in cells (Burroughs et al., 1994, Chuma et al., 1992) and will be investigated in this study as an antigen delivery and presentation system.

Unlike traditional vaccines that generally induce a predominantly humeral-mediated immunity (Amanna et al., 2008, Plotkin, 2008), the new generation of vaccines may be used to induce strong cellular-mediated immunity, important for the eradication of intracellular pathogens, such as HIV, and the support of the humeral immune response. The correlates of a protective immune response will now be discussed.

1.2 The host immune response to pathogens

The immune response is composed of two parts, an innate or non-specific response and an adaptive or antigen-specific response (Roitt & Delves, 2006). The innate immune system is the first internal defense mechanism encountered by a pathogen and can be activated within minutes of the initial invasion. This type of immunity protects the host in a non-specific manner by a combined action of specialized immune cells (Dendritic cells (DC), macrophages and natural killer cells) and soluble factors (complement and cytokines) (Parkin & Cohen,

2001). Dendritic cells are professional antigen presenting cells (APC) that are activated by microbial-associated antigens binding to their surface pattern recognition receptors (Akira & Takeda, 2004, Wilmanski et al., 2008). Micro-organisms are subsequently engulfed, their antigens processed and the resulting peptides presented on major histocompatibility complex I and II for the initiation and direction of the adaptive immune response (Hoebe et al., 2004). Unlike the innate immune response, the activation of the adaptive immune response results in the generation of immunological memory which protects the host against future challenges from the same or a similar pathogen (Castellino et al., 2009).

The adaptive immune response may be tailored to the type of microorganism, its route of entry and the site within the body where the pathogen resides, in order to elicit the most effective response (Mortellaro & Ricciardi-Castagnoli, 2011). Humeral immunity, mediated by antibody produced via B-cells, is elicited against viruses and bacterial toxins in bodily fluids in order to neutralize these entities, thus limiting the spread of infection and damage, as well as to increase the opsonized entities susceptibility to internalization and degradation by macrophages recognizing the Fc region of the antibody. Cellular immunity, mediated by T cells, is directed towards the eradication of intracellular pathogens and the support of the humeral immune response. The humeral immune response is initiated when B cells are stimulated by the B-cell receptor recognition of a specific antigen and differentiate into plasma cells that produce high affinity antibodies. $CD4^+$ T type 2 helper cells (Th2) activate B cells to produce antibody. T helper 2 cells, which form part of the cellular immune response, are stimulated via T-cell receptor recognition of specific peptides presented by major histocompatibility class II molecules (MHC II) on the surface of dendritic cells. In addition to the Th2 helper cells, $CD4^+$ T type 1 helper cells (Th1) activate macrophages to engulf and degrade pathogen-containing cells while $CD4^+$ T type 17 helper cells (Th17) activate neutrophils and induce secretion of antimicrobial peptides by epithelial cells. Cytotoxic cells (CTL) are stimulated via T-cell receptor recognition of specific peptides presented by major histocompatibility class I molecules (MHC I) on the surface of dendritic cells. These $CD8^+$ cells function by directly destroying infected cells. The dominant type of immune response elicited, Th versus CTL, is dependent on the class of microorganism that is recognized by the surface pattern recognition receptors of dendritic cells (Mortellaro et al., 2008). Toll-like receptors that are found on the plasma membrane and in endosomal compartments of dendritic cells recognize components of bacteria, viruses, protozoa and fungi (Akira & Takeda, 2004). The nucleotide-binding domain, leucine rich containing (NLR) protein family, localized to the cytoplasm in dendritic cells, recognizes viral nucleic acid or bacterial products (Wilmanski et al., 2008). The mode of presentation of an antigen to the immune system (vaccine design) must therefore be tailored to the induction of an immune response that will eliminate the targeted pathogen. Protein-based vaccines normally elicit a predominantly antibody-based immunity (Amanna et al., 2008, Plotkin, 2008) while DNA-delivered antigens are synthesized intracellularly and presented on MHC I molecules leading to the activation of a CTL response (Arrode-Bruses et al., 2010, McConkey et al., 2003). These, as well as additional, new generation vaccines will now be discussed.

1.3 Peptide-based vaccines

Immunologically relevant proteins from many pathogenic agents have been expressed *in vitro* (Mortellaro & Ricciardi-Castagnoli, 2011). Peptide vaccines, which direct the immune response towards one or a few immunogenic epitopes presented on a synthetic peptide, have been suggested as an alternative to these recombinant protein subunit vaccines (Brun et al., 2011, Purcell et al., 2007). The advantages of synthetic peptides include ease of storage, transport and distribution, ease of characterization as well as the option of introducing swift changes in the design of the peptide vaccines, particularly advantageous when dealing with rapidly mutating viruses such as influenza and HIV. However, due to the poor stability and immunogenicity of simple peptides (Tam, 1996), it is required that they be presented to immune system in conjugation with larger carrier proteins such as glutathione S-transferase (GST), bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) (Dong et al., 2006, Liu & Chen, 2005). Specific synthetic peptides have recently been found to protect mice against virulent influenza A challenge (Miller et al., 2011) and Enterohemorrhagic *Eschericia coli* infection (Wan et al., 2011).

The first successful veterinary peptide vaccine targeted canine parvovirus (Langeveld et al., 1994). Early studies on the development of a FMDV synthetic peptide vaccine were not successful as peptides spanning epitopes from the capsid VP1 protein afforded limited protection in natural hosts (DiMarchi et al., 1986, Taboga et al., 1997). The lack of T-cell helper epitopes was suggested as contributing to the failure of these peptide vaccines and these epitopes were subsequently identified in other FMDV proteins (Blanco et al., 2001, Blanco et al., 2000, Garcia-Briones et al., 2004). Protection of pigs against FMDV challenge was achieved following vaccination with a dendrimeric peptide containing a B-cell epitope with a T-helper immunodominant epitope (Cubillos et al., 2008). The multimerization of these epitopes within this dendrimeric construct, composed of multiple branched monomers originating from a central core, is thought to have enhanced the immunogenicity of the peptides and peptide dendrimers have also been used to induce immune responses against Hepatitis B, Herpes simplex, bovine leukemia and bluetongue viral peptides (Sadler & Tam, 2002). Recently, three copies each of peptides aa141-160 and aa200-213 of FMDV VP1, previously found to be immunogenic (Bittle et al., 1982, Morgan & Moore, 1990), were conjugated to swine immunoglobulin G heavy chain constant region (scIgG) and the multiepitope vaccine was found to completely protect pigs against virulent homologous FMDV challenge (Shao et al., 2011).

Peptide-based vaccines based on repeats of ELDKWA, a potent neutralizing epitope on HIV-1 gp41, were found to induce potent epitope-specific antibody responses, much stronger than in comparison with envelope glycoprotein gp160 (Chen et al., 1999, Xiao et al., 2000b). A multi-epitope vaccine based on a combination of different epitopes could elicit broadly cross-reactive antibodies (Liu et al., 2003) and as high epitope density is an important trigger of B-cell activation (Brun et al., 2011, Jegerlehner et al., 2002), three repeats each of HIV-1 neutralizing epitope ELDKWA on gp41 and epitope GPGRFY on gp120 were conjugated to GST (Li et al.,

2002). The high titers of epitope-specific antibodies elicited by this multi-epitope vaccine in mice could recognize cells which expressed the HIV-1 envelope glycoprotein on the cell surface, indicating the ability of the antibodies to recognize and bind to the natural envelope of HIV-1. A recombinant immunogen with high density epitopes of ELDKWA and ELDEWA (8 copies each) induced high titers of antibodies recognizing both epitopes on HIV-1 gp41 (Liu et al., 2005). These authors found that the use of predefined spacers between epitopes on a multi-epitope peptide impacted the level of antibody titer elicited by the epitope-vaccines. The epitopes discussed thus far have all been B-cell epitopes, capable of inducing neutralizing antibodies. CTL epitopes have also been introduced into epitope vaccines (Xiao et al., 2001). Multi-specific CTL responses have been successfully generated by a multivalent human CTL peptide construct (Sundaram et al., 2003), as well as a HIV peptide cocktail, in mice (Nehete et al., 2003, Nehete et al., 2005). Disease and viral load in macaques was also reduced after mucosal immunization with a multi-epitope HIV/SIV peptide-based vaccine containing HIV-env Th epitopes and SIV Gag or pol epitopes (Belyakov et al., 2001). By linking the multi-epitope peptide to a carrier peptide, cytotoxic T-cell responses in mice have been induced (Dakappagari et al., 2005). It is, however, difficult in most cases for peptide-based vaccines to induce effective cellular immune responses (Liu et al., 2003). The cost associated with the manufacture of these conjugated vaccines is also prohibitively expensive (Chackerian, 2007). DNA-based vaccines, which are stable, cheap to manufacture and capable of eliciting a CTL response (Arrode-Bruses et al., 2010, McConkey et al., 2003) are a feasible alternative for developing countries.

1.4 DNA vaccines

The principle of DNA vaccination was first described in 1993 (Ulmer et al., 1993). Plasmid DNA encoding an antigen under the control of a eukaryotic promoter is injected into a target cell. By using the host cells machinery the foreign protein is expressed, undergoes post-translational modifications and is presented in its native conformation to the host immune system. The bacterial origin of plasmids ensures that the presence of unique prokaryotic structural elements, such as unmethylated CpG motifs, elicits the mammalian, avian and fish innate immune response (Bauer et al., 2008, Mutwiri et al., 2003). As the antigen is produced intracellularly, this mode of antigen delivery mimics the replication of live viral pathogen and a cellular immune response is elicited. In addition to being easy and cheap to produce, DNA vaccines are also safe, as at no stage does one work with infectious agents, as well as specific, as the immune response against the nucleic acid backbone is weak (Brun et al., 2011). The immune response is elicited against only the expressed encoded antigen which allows the differentiation of infected from vaccinated animals and DNA vaccines could be used as a previously mentioned DIVA vaccines (van Oirschot, 2001). Thus DNA vaccination has been welcomed as one of the most promising methods of immunization and successful vaccines against various animal and human pathogens have, and continue, to be produced (Dhama et al., 2008, Liu, 2011, van Drunen Littel-van den Hurk et al., 2004).

A DNA vaccine encoding multiple HIV CD4 epitopes has recently been found to elicit vigorous, long-lived CD4⁺ and CD8⁺ T cell responses in mice (Rosa et al., 2011) while a mixture of DNA fragments from the conserved HIV

gag gene elicit broader T cell response than the full length gag DNA (Liu et al., 2011). A DNA vaccine, encoding the prM/E structural proteins of dengue-4 virus, was found to protect mice against homologous lethal challenge with this virus and a safety and immunogenicity of a dengue-1 DNA vaccine has recently been evaluated in human subjects in a Phase 1 clinical trial (Beckett et al., 2011). A West Nile Virus DNA vaccine has also been evaluated in a human Phase 1 clinical trial and was found to elicit neutralizing antibodies and T-cell responses (Ledgerwood et al., 2011). DNA vaccines have also been found to protect cattle against bovine viral diarrhea (BVDV) (Liang et al., 2005, Nobiron et al., 2003) and pigs against classical swine fever virus (CSFV) (Andrew et al., 2000, Ganges et al., 2005) and pseudo rabies virus (PRV) (Dory et al., 2005, Gravier et al., 2007). The potential of DNA vaccines against avian influenza, using plasmids encoding the hemagglutinin (HA) protein alone or with the nucleoprotein (NP), has also been demonstrated (Laddy et al., 2007, Rao et al., 2008). Mice, immunized with a DNA vaccine encoding avian influenza H3/H1 hemagglutinin (HA) antigens, as well as multiple epitopes from the H5/H7/H9 subtypes, elicited humoral and cellular immunity and were protected from lethal challenge with a H1N1 influenza strain (Tan et al., 2010). Indeed, DNA vaccines may even confer protection based solely on the cellular immunity as found when immunized mice were protected against foot-and-mouth disease (FMD) virus challenge even in the absence of FMDV-neutralizing antibodies (Borrego et al., 2006).

However, not all DNA vaccines are this successful. Delivery of a plasmid encoded outer capsid VP2 protein of African horsesickness virus elicited a very poor immune response in a horse (Romito et al., 1999). One of the main disadvantages of DNA vaccines is that large quantities of DNA are required in order to achieve a strong immune response (Brun et al., 2011). Various strategies have been used to improve the immune response to DNA vaccines (Dhama et al., 2008, van Drunen Littel-van den Hurk et al., 2004). These include delivering the antigen in combination with immunostimulatory cytokines such as IL-2, IL-18 or GM-CSF (Dory et al., 2009, Hanif et al., 2010, Schautteet et al., 2011, Siva Reddy et al., 2010, Zheng et al., 2011), combining the plasmid with immunostimulatory adjuvants such as a cationic lipid adjuvant (Pan et al., 2008, Smith et al., 2010) and enhancing plasmid uptake via electroporation as a mean of plasmid administration (Dolter et al., 2011, Dupuy et al., 2011, van Drunen Littel-van den Hurk et al., 2008, Xu et al., 2011a). Antigens have also been fused with immunostimulatory proteins such as the heat shock protein 70 (Uto et al., 2011) and transport proteins such as Marek's Disease Virus type 1 VP22 (Moeini et al., 2011) or the Bovine Herpes virus type 1 VP22 (Yao et al., 2009) which facilitate the movement of the fusion protein to neighbouring cells.

DNA vaccines are also routinely used to prime the immune system in heterologous 'prime-boost' strategies (Radošević et al., 2009). This principle is based on the observation that priming with an antigen presented in one form followed by a boost with the same antigen presented in a different form leads to an superior immune response to the antigen (Plotkin, 2009). DNA priming, followed subsequently by HIV envelope protein boosting, elicited high titers of neutralizing antibodies that correlated with protection against SHIV (Simian-HIV chimeric virus) challenge in rhesus macaques (Bogers et al., 2008, Pal et al., 2006). A recent study indicates that DNA env priming and gp120 protein boosting, with the appropriate mucosal adjuvant, induces mucosal antibody and

T-cell responses that could block HIV-1 mucosal transmission (Cristillo et al., 2011). Cattle, primed with DNA encoding the FMDV VP1 capsid protein and subsequently boosted with the recombinant VP1 protein or the inactivated FMDV vaccine particle, elicited an enhanced neutralizing and Th1 immune response against FMDV (Jin et al., 2005a). This strategy also elicited broadly cross-reactive neutralizing antibodies in pigs capable of neutralizing both homologous as well as heterologous FMDV serotypes (Li et al., 2008a). Although this may be due to the ability of DNA vaccines to increase the diversity of the CD4⁺ T cell response (Wu et al., 2005), a phase I clinical trial showed that human volunteers, primed with a HIV env-gag DNA and boosted with env gp120 trimer protein, did not elicit neutralizing antibodies effective against heterologous HIV isolates (Spearman et al., 2011). In addition to boosting with protein, DNA priming may also be boosted with a viral vector encoding the same proteins (Harari et al., 2008) or virus-like particles (VLPs) displaying the same proteins (Chen et al., 2010).

Besides the suboptimal immune responses elicited by DNA vaccines, risks include non-specific immune suppression, observed in mice and cattle co-immunized with FMDV DNA and a related protein vaccine (Jin et al., 2005b), and disease exacerbation if the wrong antigen is selected (Heinen et al., 2002). These factors may be overcome by the use of nonpathogenic live recombinant vectors as a means of delivering genes into target cells.

1.5 Live recombinant viral vectors

Plotkin et al., 2009 defined live recombinant vectors as 'nonpathogenic vehicles into which genes from pathogens are inserted and from which those genes are expressed'. Foreign genes encoded by a viral vector are expressed using the host cells machinery. Viral vectors span a large variety of viruses and include, amongst others, Adenoviruses and Poxviruses (Matthews, 2011).

The replication-deficient adenovirus vectors are able to elicit a broad antibody response and are powerful inducers of cellular immunity. It is not surprising that they have been used to express the antigens of pathogens such as malaria (Hill et al., 2010, Miyata et al., 2011), tuberculosis (Radošević et al., 2007, Ronan et al., 2010), Ebola (Ledgerwood et al., 2010, Sullivan et al., 2006) and HIV (Jaoko et al., 2010, Migueles et al., 2011). The capsid proteins of foot-and-mouth disease virus (FMDV), co-expressed by a recombinant adenovirus, have been found to elicit neutralizing FMDV antibodies in pigs (Wu et al., 2003) and protected cattle against virulent homologous FMDV challenge (Pacheco et al., 2005). Although many recent studies have utilized the human adenovirus serotype 5 in the development of vaccines against influenza (Tutykhina et al., 2011), measles (Lobanova et al., 2010) and Western equine encephalitis (Swayze et al., 2011), widespread pre-existing immunity to this serotype in humans and non-human primates may limit this vectors efficacy in these populations (Catanzaro et al., 2006, McCoy et al., 2007). To overcome this limitation, several alternative adenovirus

serotypes, that occur infrequently within the human population, are currently under investigation (Abbink et al., 2007, Geisbert et al., 2011, Patel et al., 2010).

Poxviruses are a large family of viruses including Avipoxviruses such as canarypox virus, Capripox virus and Orthopoxviruses such as modified vaccinia Ankara (MVA) (Gomez et al., 2011, Weli & Tryland, 2011). These vectors do not replicate in mammalian cells and can therefore be safely given even to immunocompromised recipients. Like adenoviruses, they can accommodate very large inserts and provide strong stimulation of the immune system against the vectored antigen. The canarypox virus vector is used in a number of commercial veterinary vaccines worldwide (Poulet et al., 2007, Zanotto et al., 2010). These include a canarypox virus vaccine expressing the prM/E genes of West Nile Virus (WNV) (Minke et al., 2004) and a canarypox virus vaccine expressing the hemagglutinin antigens of two strains of H3N8 equine influenza virus (Minke et al., 2007). Both these equine vaccines elicit pathogen-specific neutralizing antibodies and a cell mediated immune response in horses (Adams et al., 2011, El Garch et al., 2008). A recombinant canarypox virus vector, co-expressing the genes encoding the VP2 and VP5 outer capsid proteins of Bluetongue virus (BTV) serotype 17, induced BTV-specific neutralizing antibodies in vaccinated sheep and afforded protection against challenge with a field strain of BTV-17 (Boone et al., 2007). Similarly, the genes encoding outer capsid proteins VP2 and VP5 of African horsesickness serotype 4, were also expressed via a recombinant canarypox virus vector and elicited AHSV-specific neutralizing antibodies, and protection against homologous virulent viral challenge, in horses immunized with this recombinant vector (Guthrie et al., 2009). Canarypox viral vectors are also being used to develop vaccines against HIV-1 (Liu et al., 2008, Pantaleo et al., 2010, Rerks-Ngarm et al., 2009).

The Capripox virus vector expressing the major core VP7 protein of Bluetongue virus serotype 1 conferred partial protection to vaccinated sheep against heterologous lethal BTV-3 challenge, in the absence of a neutralizing antibody response (Wade-Evans et al., 1996). This suggested the induction of a cellular immune response by the VP7 protein. A combination of recombinant capripox virus viruses, expressing the capsid proteins VP2 and VP7 and non-structural proteins NS1 and NS3 proteins of BTV-2, however, also only conferred partial protection of sheep against homologous virulent BTV-2 challenge (Perrin et al., 2007).

Another poxvirus vector is the vaccinia virus, however due to its ability to replicate within mammalian cell lines and its rare but serious adverse side-effects in immunocompromised individuals, attenuated and/or modified strains of this vector have been proposed for vaccine development (Kennedy & Greenberg, 2009, Yilma et al., 2010). Although a recombinant vaccinia virus, expressing the VP2 protein of AHSV serotype 4, induced AHSV neutralizing antibodies and afforded protection against homologous challenge in horses (Stone-Marschat, 1996) and a recombinant vaccinia vector, expressing BTV-1 VP2 antigen, induced protective immunity against virulent homologous BTV-1 challenge in sheep (Lobato et al., 1997), this approach is most likely not to be pursued due to the vectors ability to replicate in vivo and possible pathogenicity in immunocompromised animals. Modified vaccinia Ankara (MVA) is an attenuated vaccinia strain that is replication incompetent in mammalian cell lines

and has been suggested as an alternative to the vaccinia vector due to its non-pathogenicity in even immunocompromised individuals (Kennedy & Greenberg, 2009, Wilck et al., 2010). Vaccination of horses with a recombinant MVA vector expressing the VP2 of AHSV serotype 4 induced a strong AHSV neutralizing humeral immune response (Chiam et al., 2009). An experimental mouse model for AHS has recently been developed that should negate most of the ethical and financial considerations associated with conducting AHSV research (Castillo-Olivares et al., 2011). The MVA-VP2 recombinant, previously shown to induce a neutralizing immune response in horses (Chiam et al., 2009), was found to protect interferon- α receptor knock out (IFNAR $^{-/-}$) mice from homologous virulent AHSV-4 viral challenge. MVA is also able to elicit a potent, long term cellular immune response which protected mice against lethal challenge with the Western Reserve strain of vaccinia virus (Green et al., 2011). Recent studies that utilize recombinant MVA are in the development of vaccines against HIV (Currier et al., 2010, Guerra et al., 2010, Howles et al., 2010), influenza A (Berthoud et al., 2011, Hessel et al., 2011) and tuberculosis (Scriba et al., 2010). Poxvirus vectors are also routinely used in heterologous prime-boost strategies in order to enhance the immune response elicited. Immunization with DNA encoding the VP2, VP5 and VP7 of Bluetongue serotype 4 followed by boosting with a recombinant MVA vector expressing the same antigens protected IFNAR $^{-/-}$ mice from homologous virulent BTV-4 viral challenge (Calvo-Pinilla et al., 2009). A DNA/MVA approach was also used to express HIV-1 virus-like particles in a Phase 1 clinical trial (Goepfert et al., 2011). MVA prime-Fowlpoxvirus (FPV) boost strategies have also recently been utilized to enhance the response against HIV antigens (Keefer et al., 2011, Soprana et al., 2011).

The Myxoma virus (MYXV), a poxvirus infecting rabbits, was used to deliver the BTV-8 VP2 and VP5 capsid proteins into sheep (Top et al., 2012). Although animals immunized with the MYXV-VP2 recombinant elicited neutralizing antibodies and were partially protected against homologous BTV-8 challenge, animals immunized with the dual recombinant MYXV-VP2-VP5 were not protected. This was unexpected as the co-expression of BTV VP2 and VP5 in a previous study enhanced the protective immune response against BTV infection (Roy et al., 1990b).

Although poxvirus vectors have been quite successful in the development of vaccines against numerous human and animal pathogens, they are difficult to produce and very high numbers of infectious units are required per dose for full efficacy (Jordan et al., 2011). Protein-based virus-like particles (VLPs), which mimic infectious virions but do not contain viral genome, may be produced in a variety of eukaryotic expression systems and eliminates the need to express foreign genes from a live recombinant viral vector.

1.6 Virus-like particles (VLP's)

Virus-like particles (VLP's) are composed of one or several recombinantly expressed viral proteins which spontaneously assemble into supramolecular structures that mimic virions, or in some cases, subviral particles (Brun et al., 2011). As they lack a viral genome, VLPs are not capable of replication within host cells and thus

represent a safe vaccine platform (Jennings & Bachmann, 2007). VLPs, like infectious virions, are composed of proteins arranged geometrically into dense repetitive arrays (Jennings & Bachmann, 2008, Noad & Roy, 2003). These structures are characteristic of microbial antigens and VLPs are thus able to elicit strong humoral and cellular immune responses against antigens presented in repetitive arrays on their surfaces (Grgacic & Anderson, 2006, Spohn & Bachmann, 2008). Their safety and efficacy have made VLPs attractive vaccine candidates for many viral diseases (Buonaguro et al., 2010, Chackerian, 2007, Jennings & Bachmann, 2008, Ramqvist et al., 2007, Roy, 2009, Spohn & Bachmann, 2008).

The recently commercialized human papillomavirus (HPV) VLP vaccine, used to vaccinate individuals against human papillomavirus serotypes known to cause cervical cancers and genital warts, consists of the HPV L1 outer capsid protein (Garland et al., 2007, McKeage & Romanowski, 2011, Paavonen et al., 2007). The human hepatitis B (HBV) VLP vaccine is also composed of a single protein, the HBV nucleocapsid (Roldao et al., 2010), as is the Norovirus VLP (Xia et al., 2007). Multiprotein VLP vaccines currently being investigated include, amongst others, a HIV-1 pr55gag-gp120 VLP (Buonaguro et al., 2002, Buonaguro et al., 2006), HIV-1 gag-CD40L VLP (Franco et al., 2011), a Norovirus VLP (Bok et al., 2011), a Marburg virus glycoprotein-VP40 matrix protein VLP (Swenson et al., 2008), an Ebola virus glycoprotein-nucleoprotein-VP40 matrix protein VLP (Warfield et al., 2007), a Lassa hemorrhagic fever glycoprotein-nucleoprotein and Z matrix protein VLP (Branco et al., 2010), a rotavirus VP2-VP6-VP7 VLP (Yang et al., 2011) as well as a Rift Valley Fever nucleocapsid-EnvG_N-Env-G_C VLP (Mandell et al., 2010, Naslund et al., 2009). In addition, an avian influenza H5N1 VLP, presenting a computationally optimized broadly reactive HA antigen, elicited broadly reactive antibodies and was found to protect mice and ferrets against challenge with a heterologous highly pathogenic avian influenza viral strain (Giles & Ross, 2011). FMDV VLPs, consisting of structural proteins VP0, VP1 and VP3, are capable of inducing neutralizing antibodies that, in conjunction with a cell-mediated immune response, are responsible for protection of cattle against virulent homologous (Li et al., 2008b) and heterologous (Li et al., 2011) FMDV viral challenge.

Baculovirus expression systems have been used to generate a wide range of VLPs (Beljelarskaya, 2011, Casal, 2001). The expression of the genes encoding the Bluetongue virus major core proteins VP3 and VP7 using a recombinant baculovirus expression system allowed for the spontaneous assembly of viral core-like particles (CLPs) in infected *Spodoptera frugiperda* insect cells (French & Roy, 1990). However, the protection afforded to vaccinated sheep against homologous virulent BTV challenge by the conserved VP3 and VP7 proteins in the form of CLPs was only partial (Roy et al., 1994b). A recent study indicated that vaccination of sheep with BTV-1 CLPs failed to prevent BTV-1 replication and the clinical manifestation of disease, although the severity of the disease and viraemia was mitigated (Stewart et al., 2012). This low level of protection afforded by the CLPs was most likely due to the cell-mediated immune response induced by VP7 (Jeggo et al., 1984, Jeggo et al., 1985, Rojas et al., 2011). Thus BTV CLPs were not considered a suitable vaccine candidate to protect animals from disease.

Authentic, double-capsid virus-like particles (VLPs) have also been synthesized via co-expression of all four of the major structural protein genes in a single cell (French et al., 1990). These VLPs were able to elicit high titers of neutralizing antibodies in guinea pigs against the homologous BTV serotype and, in doses of 10µg or more, were also found to elicit protective responses in sheep against bluetongue infection (Roy et al., 1992). Vaccination with VLPs showed long-lasting protection against homologous BTV challenge and provided some evidence for cross-protection against heterologous virus, depending on the challenge virus and amounts of antigen used for protection (Roy et al., 1994a). Stewart et al., 2012 also found that sheep vaccinated with BTV-1 VLPs developed a neutralizing antibody response that inhibited replication of the challenge BTV-1 virus and prevented clinical disease manifestation and viraemia. For decades twenty four distinct BTV serotypes were thought to be responsible for causing bluetongue disease in ruminants, however, recently two further BTV serotypes, BTV-25 (Hofmann, 2008) and BTV-26 (Maan, 2011), have been identified, on the basis of VP2 sequence analysis and subsequently on the neutralizing activity of VP2, in goats and sheep, respectively. One of the disadvantages of the BTV VLP strategy was that the expression of all four proteins from each serotype was required for the formation of serotype-specific VLPs. In order to simplify the synthesis of VLPs of multiple BTV serotypes, the genes encoding the conserved inner capsid proteins VP3 and VP7 were preintegrated into the baculovirus genome (Stewart et al., 2010). Thus only the genes encoding the serotype-specific VP2 and VP5 proteins would have to be manipulated in order to synthesize BTV VLPs of a desired serotype. VLPs of BTV serotypes 2, 4 and 9 were synthesized using this system and were found to be morphologically identical to BTV VLPs previously reported (Belyaev & Roy, 1993, French et al., 1990). In addition, sheep vaccinated using the BTV-2 VLPs were protected from infection with virulent BTV-2 challenge virus. A cocktail of VLPs containing the VP2 and VP5 proteins of different BTV serotypes may be used as a multivalent vaccine to elicit multiple serotype-specific or cross protective immune responses. Although a polyvalent vaccine consisting of a mixture of BTV serotype 1, 2, 10, 13 and 17 VLPs had previously been found to protect, or partially protect, sheep against challenge with homologous or heterologous virulent BTV virus, respectively (Roy et al., 1994a), a recent study indicates the combination of VLPs of certain serotypes may interfere with BTV vaccine efficacy (de Diego et al., 2011).

Although BTV VLPs are relatively easy to synthesize in large quantities using the baculovirus expression system, co-expression of AHSV-9 VP2, VP5, VP3 and VP7 proteins yields very low levels of AHSV VLPs (Maree, 2000). Co-expression of AHSV-9 VP7 and VP3 proteins in insect cells also yields a low levels of AHSV core-like particles (CLPs), with the VP7 protein assembling preferentially into large, distinctive crystal-like particles in the cytoplasm of the infected cells (Maree et al., 1998), resembling those previously observed in recombinant baculovirus-infected insect cells (Chuma et al., 1992). The insoluble nature of AHSV VP7 is thought to be responsible for the assembly of this protein into crystal-like structures in preference to core-like particles (CLPs) with AHSV VP3 (Maree, 2000) and thus a AHSV CLP or VLP based vaccine strategy would not be viable.

The BTV outer capsid VP2 protein is the host's main target for viral neutralizing activity and also contains T-cell epitopes (reviewed in Top et al., 2012). It is thus not surprising that, when purified from virus particles, this protein was found to elicit protective immunity in vaccinated sheep (Huismans et al., 1987a, Roy et al., 1990b). These initial studies also indicated that co-expression of VP2 with VP5 in baculovirus-infected insect cells enhanced the protective immune response against BTV infection (Roy et al., 1990b). The co-expression of these two proteins from a recombinant myxomavirus recombinant vector has, however, been shown to elicit lower levels of protection against virulent BTV-8 challenge in sheep than the VP2 protein on its own (Top et al., 2012). Immunization with a recombinant vaccinia virus, encoding the BTV-1 VP2 and VP5 proteins, was also shown to protect only four out of six sheep against homologous BTV-1 challenge while sheep vaccinated with the vaccinia virus encoding the VP2 protein protected all the challenged sheep (Lobato et al., 1997). In addition, a mixture of capripoxviruses expressing the VP2, VP7, NS1 and NS3 proteins conferred limited protection against homologous BTV-2 challenge (Perrin et al., 2007). It has been hypothesized that the limited protection induced by BTV VP2, when co-expressed with other BTV proteins in a poxviral expression system, may be due to lower VP2 protein levels (Top et al., 2012). However, AHSV-4 VP2 protein, or a combination of VP2 and VP5 of this serotype, expressed in recombinant baculovirus-expressed infected insect cells failed to induce neutralizing antibodies and protection following virulent AHSV-4 challenge in immunized horses (Martinez-Torrecuadrada et al., 1996). This is most likely due to the fact that the majority of the AHSV VP2 protein aggregates when expressed in a heterologous expression system (du Plessis et al., 1998). The small soluble AHSV VP2 fraction that elicits neutralizing antibodies is most likely responsible for the partial protection of horses against virulent AHSV challenge (du Plessis et al., 1998), as well as for the complete protection of horses by the baculovirus-expressed AHSV-5 VP2 (Scanlen et al., 2002) and AHSV-4 VP2 (Roy et al., 1996a) proteins. The induction of a protective, neutralizing immune response in horses by a combination of the baculovirus-expressed AHSV-4 VP2, VP5 and VP7 proteins indicated that the VP7 protein may assist with the correct VP2 protein folding and improve protein solubility (Martinez-Torrecuadrada et al., 1996).

An alternative to the use of a full-length protein, which may be insoluble when expressed in a heterologous system, is the use of a small portion of an antigen, such as an immunologically important epitope, which would concentrate the immune response against this desired portion of the antigen (Tissot et al., 2010). Small antigenic peptides are commonly not stable and display a poor immunogenicity (Tam, 1996). The insertion of such peptides into viral structural proteins to generate chimeric particles is a method for displaying foreign peptides in order to induce an immune response (Brun et al., 2011). VLPs are thus modified to function as molecular scaffolds for the presentation of foreign antigens. Viruses from 14 different families have been used for the production of such chimeric VLP particles (Chackerian, 2007, Grgacic & Anderson, 2006). The nucleocapsid of hepatitis B virus, HBcAg, is a commonly used carrier for foreign epitopes (Pumpens & Grens, 2001, Pumpens, 2008). Multiple dimers of the single core protein form a highly symmetric structure, with the repetitive antigen resulting in an enhanced immunogenicity (Jegerlehner et al., 2002, Jennings & Bachmann, 2008). Various epitopes have been inserted and presented on fusion hepatitis B virus core particles including,

most recently, Hepatitis B virus and Hepatitis C virus epitopes (Sominskaya et al., 2010), foot-and-mouth disease viral epitopes (Zhang et al., 2007), *Bacillus anthracis* (Anthrax) epitope (Yin et al., 2008), a hantavirus epitope (Geldmacher et al., 2005) and *Plasmodium falciparum* circumsporozoite (Malaria) epitopes (Gregson et al., 2008). Virus-like particles (VLPs) formed by the structural proteins of other viruses have also been used to present foreign epitopes to the immune system. Recent examples include Newcastle Disease (NDV) virus-like particles containing respiratory syncytial virus (RSV) G protein (Murawski et al., 2010), Influenza A VLPs displaying a peptide from *Mycobacterium tuberculosis* (Krammer et al., 2010), Rotavirus-like particles displaying a simian paramyxovirus epitope (Peralta et al., 2009) and Human papillomavirus-like particles displaying Chlamydia trachomatis epitopes (Xu et al., 2011b). Insertion of antigenic peptides into the capsid proteins of adenovirus VLPs has recently been extensively reviewed (Matthews, 2011). Bluetongue virus VLPs and CLPs have also been used to display foot-and-mouth disease and influenza A viral epitopes (Adler et al., 1998, Roy & Sutton, 1998), a Hepatitis B viral epitope (Belyaev & Roy, 1992), the enhanced green fluorescent protein (eGFP) (Kar et al., 2005), a rabies viral epitope (Le Blois & Roy, 1993) and a bovine leukemia virus (BLV) glycoprotein epitope (Tanaka et al., 1995) to the immune system.

In order to induce a good humeral immune response, foreign antigens or peptides must be displayed in immunodominant regions on the surface of VLPs at a high density (Brun et al., 2011). It is, however, not possible to predict whether an antigenic peptide, inserted into a particular immunodominant region, will interfere with protein folding and affect VLP assembly or if it will be presented on the surface of VLP in its immunogenic conformation. In addition, the size of the epitope that can be inserted into VLP immunodominant domains is limited (Janssens et al., 2010, Peralta et al., 2009, Tanaka et al., 1995, Tissot et al., 2010). VLPs containing peptides longer than 20 amino acids often fail to assemble. *In vitro* conjugation to a VLP particle (Jegerlehner et al., 2002) may also be a way of displaying larger peptides to the immune system as the peptide may be able to fold into its native, immunogenic conformation without interfering with VLP folding and assembly. However, as mentioned previously, the cost associated with the manufacture of conjugated vaccines is also prohibitively expensive (Chackerian, 2007). The presentation of epitopes via particulate delivery systems such as the Norovirus P particle (Tan et al., 2011), the Bluetongue NS1 tubule (Murphy & Roy, 2008) and African horsesickness VP7 crystal like-particles (Maree, 2000) may be an alternative to the use of VLPs.

1.7 Orbiviral protein particles as antigen delivery systems

Bluetongue virus (BTV) and African horsesickness virus (AHSV) are closely related orbiviruses in the family *Reoviridae*, with BTV being the prototype orbivirus (Holmes et al., 1995). These viruses possess non-enveloped virions, encapsidating 10 segments of double-stranded RNA genome (Roy, 1996b). The large (86nm in diameter), icosahedral virion is composed of seven structural proteins forming a double-layer capsid (Burroughs et al., 1994, Huismans et al., 1987b, Mertens et al., 1987, Oellermann et al., 1970, Van Dijk & Huismans, 1988). The major structural proteins VP2 and VP5 form the outer capsid while the major structural proteins VP3 and

VP7 form the inner and outer layers of the inner capsid, or core, respectively, (Bremer, 1976, Burroughs et al., 1994, Laviada et al., 1993, Mertens et al., 1987, Verwoerd & Huismans, 1972, Verwoerd et al., 1979, Zhang et al., 2010) (Fig 1.1). The three minor structural proteins VP1, VP4 and VP6 have RNA polymerase and RNA-modifying properties (Boyce et al., 2004, Kar & Roy, 2003, Matsuo & Roy, 2009, Ramadevi et al., 1998, Roy, 2008a) and are closely associated with the ten double stranded genome segments within the viral core particle (Bremer et al., 1990, Stuart et al., 1998, Verwoerd et al., 1972). The ten genome segments have, until recently, been thought to encode the seven structural (VP1-VP7), as well as three nonstructural proteins, NS1, NS2, NS3/NS3a which play a role in viral assembly, transport and exit from the cell (Brookes et al., 1993, Meiring et al., 2009, Roy, 2008b, Roy et al., 1990a). A fourth non-structural protein, NS4, has recently been found to be encoded by an open reading frame in segment 9, overlapping the VP6 open reading frame (Firth, 2008), is related to proteins that bind nucleic acids or are associated with membranes (Belhouchet, 2011) and is thought to have an important role in virus-host interaction (Ratinier et al., 2011). The non-structural protein 1 (NS1) of Bluetongue virus and structural viral protein 7 (VP7) of African horsesickness virus are proposed as particulate antigen delivery systems (Mikhailov et al., 1996, Wade-Evans et al., 1997).

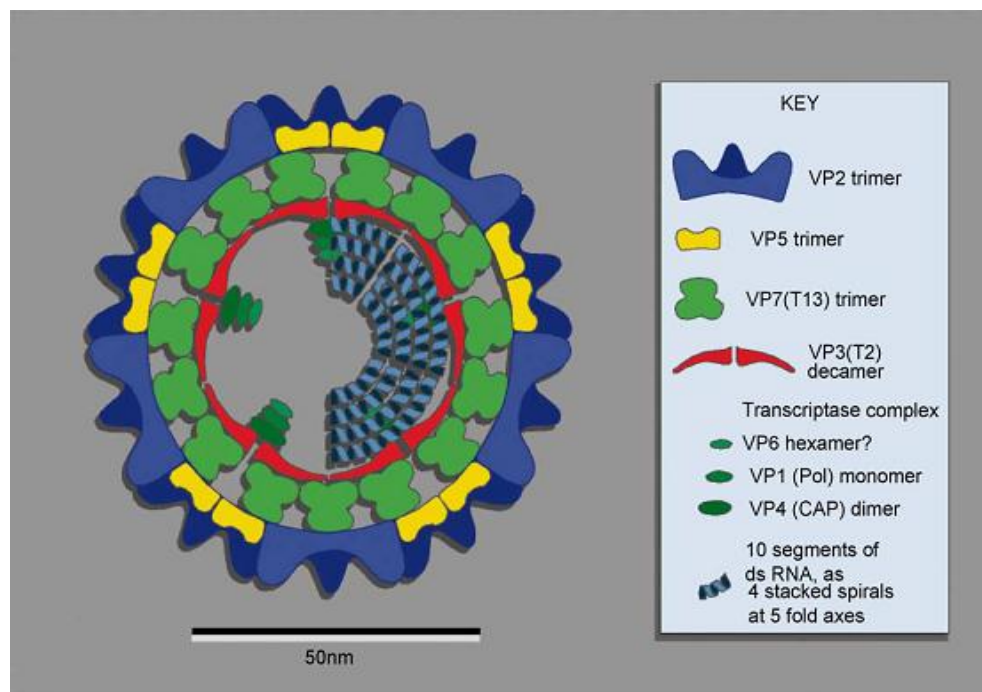


Fig 1.1: Schematic diagram of the structure of the BTV virion (Mertens, 2000)

1.7.1 Bluetongue virus NS1 tubules

The nonstructural protein 1 (NS1) is the most abundantly synthesized BTV protein (Huismans & Els, 1979) and multiple copies of this protein spontaneously assemble into particulate helical tubules in the cytoplasm of BTV-

infected cells or recombinant baculovirus-infected insect cells (Mikhailov et al., 1996). NS1 is proposed to be involved in BTV pathogenesis and in the augmentation of virus-cell association (Owens et al., 2004). The first ten amino acids of the N terminal of the protein, as well as ten or more of the carboxyl terminal amino acids, are essential for tubule formation (Monastyrskaya et al., 1994, Monastyrskaya et al., 1995). An antigenic site was identified near to the carboxyl terminus of the NS1 protein and is thought to be displayed in regular and repeating arrays on the surface of the helical NS1 tubules (Monastyrskaya et al., 1995). The NS1 protein has subsequently been manipulated to carry foreign antigenic sequences at the C-terminus of BTV NS1 with the thousands of copies of the foreign immunogen, displayed on the surface of the undisrupted tubular structures in regular and repeating arrays, stimulating both humeral and cellular immunity (Murphy & Roy, 2008). The 44 amino acid sequence from *Clostridium difficile* toxin A, the 48 amino acid sequence from the hepatitis B virus preS2 region and the 116 amino acid bovine leukemia virus p15 protein were all displayed on the surface of the tubules, expressed in recombinant baculovirus-infected insect cells, and were highly immunogenic (Mikhailov et al., 1996). This is not surprising as many thousands of copies of the foreign epitopes are arranged on the surface of self assembled NS1 particulate tubules in a highly ordered and repetitive manner resulting in enhanced epitope immunogenicity (Jennings & Bachmann, 2008, Schiller et al., 2008). The presentation of a CD8⁺ T cell epitope from lymphocytic choriomeningitis virus (LCMV) nucleoprotein on chimeric NS1 tubules induced a strong cytotoxic T lymphocyte responses (CTL) response in mice and protected the immunized mice from infection with a lethal challenge of LCMV (Ghosh et al., 2002b). In addition, chimeric NS1 protein with a FMD VP1peptide (aa 135-144) was also able to elicit a potent humeral immune response that could protect immunized mice from virulent FMDV challenge (Ghosh et al., 2002a). A chimeric BTV immunogen, presenting the HIV-1 subtype A gag protein and a string of cytotoxic T cell epitopes (527 amino acids in total), did not significantly interfere with the inherent tubule formation of NS1 and induced HIV-1 specific T cells (Larke et al., 2005).

The NS1 tubules formed by AHSV have been characterized, and although they have a lower sedimentation (S) value than BTV tubules, have a diameter that is half the width of the BTV tubules and lack a ladder like structure (Maree & Huismans, 1997, van Staden et al., 1998), they, nonetheless, possess potential for the display of immunogenic epitopes to the immune system and should be investigated further.

1.7.2 African horsesickness virus VP7

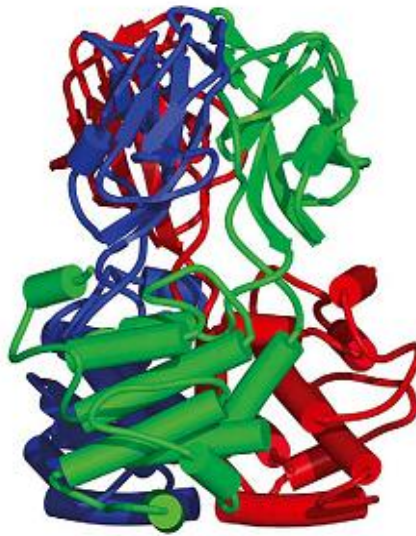
The trimers formed by the AHSV VP7 protein are proposed as a general purpose vaccine delivery system for the presentation of antigenic peptides. Viral protein 7 (VP7) is one of the major structural proteins forming the orbivirus core (Fig 1.1).

1.7.2.1 VP7 and orbivirus core structure

The VP7 monomer consists of a top domain, containing the central one-third of the polypeptide chain (aa121-249) folded into an antiparallel β -sandwich, and a bottom domain, containing the N-terminal region composed of 5 α -helices (aa1-120) and four C-terminal α -helices (aa250-349) together forming a bundle of nine helices with loops in between (Grimes et al., 1995) (Fig 1.2B). Three VP7 monomers interact extensively to form a trimer (85Å long) with a relatively flat base (65Å) (Grimes et al., 1995, Grimes et al., 1998). Each VP7 monomer is twisted so that the top domain of one monomer rests on the bottom domain of a threefold related subunit (Fig 1.2A). Two hundred and sixty of these trimers form the orbivirus core surface. The local threefold axis of the trimer is perpendicular to the core surface and the broad, flat base of each trimer, composed of bottom domains, interacts hydrophobically with the inner VP3 subcore at 13 different sites (Grimes et al., 1995, Grimes et al., 1998, Grimes et al., 1997, Limn & Roy, 2003). The apex of the trimer, composed of top domains, interacts extensively with the outer capsid proteins VP2 and VP5 (Nason et al., 2004). The surface of the icosahedral 70nm core consists of 13 copies of the VP7 molecule (~38 kDa) in the form of four trimers (P, Q, R and S) in general positions and one (T) located with its threefold axis aligned with the icosahedral threefold axis (Grimes et al., 1998, Grimes et al., 1997, Prasad et al., 1992) (Fig 1.3A). The underlying scaffold is made up of 120 copies of VP3 (~103 kDa) that are arranged at T=2 icosahedral symmetry within the subcore layer (Fig 1.3B). The assembly of the VP7 trimers on the VP3 subcore is initiated at multiple preferred sites with the 'T' trimers (found on the three-fold axis of the icosahedron) attaching to the VP3 scaffold first with the remaining trimers, which interact more weakly with VP3, being sequentially added to fill the gaps between the stronger interactions (Limn & Roy, 2003).

The base of the trimer is responsible for the interactions between the trimer and the underlying scaffolding layer of VP3 as well as the interactions with other trimers within the T=13 icosahedral lattice (Grimes et al., 1995). The substitution of key conserved amino acid residues within the α -helices in the bottom domain of VP7, although still allowing the formation of trimers, affected both the VP7-VP3 interactions necessary for successful core-like particle assembly and VP7 trimer-trimer interactions (Limn & Roy, 2003, Limn et al., 2000).

A



B

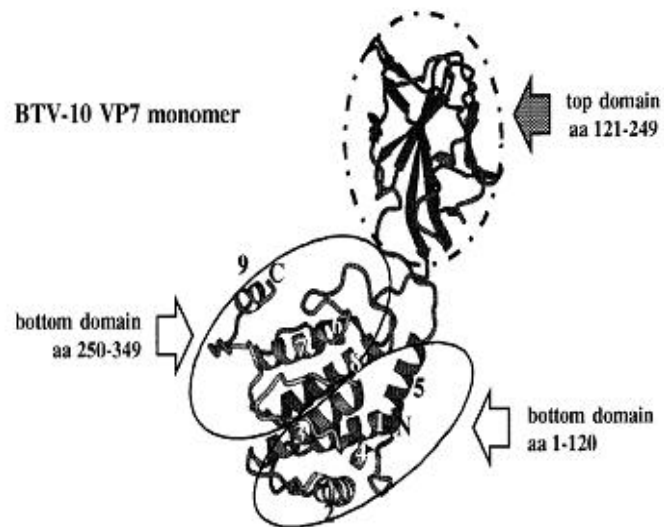
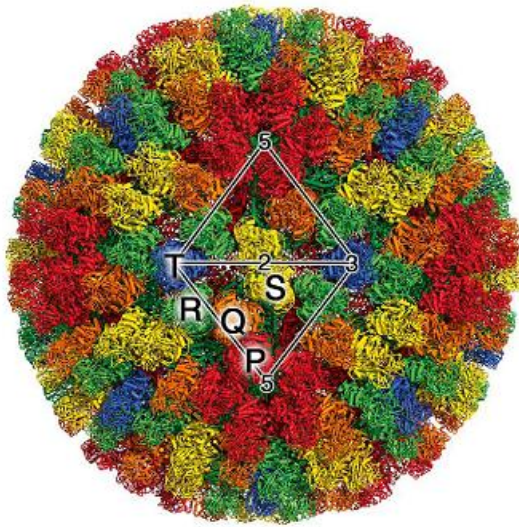


Fig 1.2: The X-ray crystallographic structure of BTV VP7 trimer (A) and monomer (B) with each monomer represented by a different colour (Grimes et al., 1995).

(A)



(B)



Fig 1.3: (A) Schematic representation of the BTV core structure. The icosahedral fivefold, threefold and twofold axes are labeled and the icosahedrally independent VP7 trimers P (purple), Q (orange), R (green), S (yellow) and T (blue) are indicated (Grimes et al., 1998) (B) The BTV subcore composed of 120 copies of VP3(T2). The icosahedral fivefold, threefold and twofold symmetry axes are labeled. The conformationally distinct VP3 A and B molecules are indicated in the colours green and red, respectively. One VP3 decamer, an assembly intermediate, is highlighted in orange (Grimes et al., 1998).

1.7.2.2 VP7 solubility

Although the BTV VP7 protein contains a high percentage of hydrophobic residues (Roy et al., 1991), BTV-10 VP7 expressed by recombinant baculoviruses in insect cells is soluble and forms trimers (Basak et al., 1992). Although AHSV VP7 shares 45% amino acid identity with BTV VP7 (Iwata et al., 1992a, Iwata et al., 1992b), when expressed by recombinant baculovirus AHSV-4 VP7 assembles into insoluble, disc-shaped crystal-like particles (Chuma et al., 1992). These flat, hexagonal crystal-like particles (5µm) were also observed in the cytoplasm of AHSV-9 infected baby hamster kidney cells (BHK) and their highly ordered and symmetric structure of these VP7 crystal-like particles is similar to that of the VP7 capsomers on the outer surface of the AHSV core (Burroughs et al., 1994). These insoluble crystal-like particles have been shown to be composed of AHSV trimers (Monastyrskaya et al., 1997). An X-ray crystallography study which determined the structure of the top domain of the AHSV-4 VP7 protein suggested that the distribution of amino acid residues in this domain may be responsible for the insolubility of AHSV VP7 (Basak et al., 1996). To determine whether this is indeed the case, chimeric VP7 proteins were constructed where the upper domain of BTV-10 VP7 was substituted with that of

AHSV-4 VP7 and vice versa (Monastyrskaya et al., 1997). The top domain of AHSV VP7 is structurally very similar to that of the BTV VP7 protein (Basak et al., 1996, Grimes et al., 1995) and they share 29% amino acid identity (Iwata et al., 1992b).

This study showed that the chimeric VP7 protein ABA, containing the BTV-10 top domain, was as soluble as WT BTV VP7, while the chimeric BAB VP7 protein, containing the AHSV-4 VP7 top domain, was insoluble. Single amino acid substitutions within the top domain of AHSV-4, when alanine (A) in position 167 was replaced by arginine (R) and phenylalanine (F) by tyrosine (T) in position 209, increased the solubility of the BAB chimeric protein but not the wild type AHSV-4 VP7 protein (Monastyrskaya et al., 1997). The AHSV alanine was replaced by arginine as this is the equivalent BTV residue in position 168 of BTV-10 VP7 and forms part of the RGD motif, responsible for BTV core attachment to a cell surface receptor (Grimes et al., 1995, Tan et al., 2001). However, the hydrophilic area where the RGD motif finds itself (aa168-176) is associated with the C-terminal helix 9 of an adjoining VP7 monomer (Basak et al., 1996). It has been suggested that the lower solubility of the AHSV-4 VP7 A167R mutant protein, when compared to the BAB A167R, is likely due to the presence of the hydrophobic leucine residue in position 345 of the C-terminal helix 9 of AHSV-4 VP7 instead of the hydrophilic arginine residue in position 345 of BTV VP7 (Monastyrskaya et al., 1997). The leucine residue in position 345 in the AHSV-9 VP7 protein was subsequently substituted with a polar, positively charged arginine residue, the BTV equivalent, and initial results indicate an increase in the solubility of the AHSV-9 VP7 protein (Meyer, 2002).

Both the above mentioned ABA and BAB chimeric VP7 proteins were found to form trimers that were stable in the presence of SDS at room temperature (Monastyrskaya et al., 1997). Although it has been suggested that the VP7 top domain plays a role in trimer formation (Grimes et al., 1995), it appears to be a minor one. The N- and C-terminals of the VP7 protein that together form the VP7 bottom domain seem to be responsible for the ability of VP7 monomers to fold correctly and form trimers (Monastyrskaya et al., 1997). The replacement of the 5 N-terminal helices in the bottom domain of the ABA chimera with those of BTV-10 resulted in incorrect folding, no trimer formation and protein aggregation. This was also observed when the 4 C-terminal helices of ABA were substituted with those of BTV-10 resulting in the ABB chimera. These results indicate that the structural differences between the N- and C-terminal helices of BTV-10 and AHSV-4 VP7 are significant enough to abolish trimer formation, even though the amino acid sequences of the AHSV VP7 and BTV VP7 bottom domains show a greater identity than the top domains, with 65% in the N terminal and 42% in the C-terminal region (Iwata et al., 1992b). Additional studies also implicate the N- and C-terminals of VP7 in trimer formation (Hosamani et al., 2010, Wang et al., 1994).

1.7.2.3 Presentation of epitopes by AHSV VP7

The conserved, group-specific VP7 protein of bluetongue virus (Gumm & Newman, 1982, Huisman & Erasmus, 1981) contains a number of B-cell and T-cell epitopes. A serogroup-specific, immunodominant and conformational B-cell epitope was identified between amino acids 30-48 on the N-terminal of BTV VP7 and was found to be accessible at the surface of intact virions and core particles (Eaton et al., 1991, Wang et al., 1996, Wang et al., 1994). Five different B-cell epitopes in other parts of BTV VP7 have also been mapped and have been found to be weakly accessible on only the core particles (Wang et al., 1996). Although immunodominant serotype cross-reactive T-cell determinants have previously been located within the structural proteins of the BTV cores (Takamatsu et al., 1992), two CD4⁺ and three CD8⁺ highly conserved T cell epitopes have recently been identified within the VP7 protein of BTV-8 (Rojas et al., 2011). A cell-mediated immune response was thought to be responsible for the partial protection of sheep, immunized with recombinant capripoxvirus expressing BTV-1 VP7 and challenged with a heterologous BTV-3 strain (Wade-Evans et al., 1996) as well as for the protection of 80-100% of mice, immunized with AHSV-9 VP7 crystal-like particles, against heterologous, virulent AHSV-7 challenge (Wade-Evans et al., 1997). However, a recent study indicates that a serotype-specific, neutralizing humoral immune response is essential for complete protection against BTV infection (Stewart et al., 2012).

Foreign antigenic peptides may also be presented to the immune system by the AHSV VP7 protein. However, the effectiveness of AHSV VP7 as an antigen display system will depend, partly, on the ability of the VP7 fusion protein to fold correctly and form trimers. As previous studies have indicated that the bottom domain of VP7 plays an integral role in trimer formation (Monastyrskaya et al., 1997), any peptide insertions should be limited to the top domain of VP7. Several positions within the top domain of the BTV VP7 protein have been identified as being good candidates for the insertion and presentation of foreign epitopes (Roy, 1996a). These included amino acid position 145 (Adler et al., 1998, Le Blois & Roy, 1993, Roy et al., 1997, Roy & Sutton, 1998) and the RGD motif (aa168-170) (Roy, 1996a), responsible for BTV attachment to a cell surface receptor (Grimes et al., 1995, Tan et al., 2001). Similarly amino acid positions 144, 177 and 200, within the top domain (aa121-249) of the AHSV-9 VP7 protein, were identified from hydrophilicity plots (Fig 1.4) as being optimally located and solvent-accessible on the surface of the AHSV VP7 trimer molecule (Maree, 2000) (Fig 1.5A). From the secondary structure predictions of AHSV-9 VP7, these positions are located in β -turns between β -sheets in the top domain of VP7 (Fig 1.5B). The arginine amino acid represents an ideal site for the insertion of foreign peptides as it forms part of the RGD motif, located on a highly flexible loop (aa175-180) (Basak et al., 1996).

AHSV-9 VP7 positions 144, 177 and 200 were subsequently modified to facilitate insertion of peptides. Six amino acids, encoded by restriction enzyme sequences, were inserted between amino acids 177-178 (Maree, 2000), amino acids 200-201 (Maree, 2000) and amino acids 144-145 (Riley, 2003) in the top domain of AHSV-9 VP7. For the simultaneous presentation of epitopes dual and triple insertion vectors were also constructed by

Riley, 2003 and van Rensburg, 2004, respectively. Although peptide insertions into the top domain may not influence trimer formation, they may result in the formation of more soluble AHSV VP7 trimers that do not assemble into VP7 crystal-like particles (Basak et al., 1996, Monastyrskaya et al., 1997). Although the AHSV VP7 crystal, consisting of many VP7 trimers assembled in a highly ordered, symmetric structure, was initially envisioned as the particulate antigen presentation system, free, soluble VP7 trimers may have an advantage due to the increased surface area available for the display of antigenic peptides to the immune system. The effect of peptide insertion into the AHSV top domain on protein solubility will be investigated in this study. In addition, although the top domain does not play an integral role in trimer formation (Monastyrskaya et al., 1997), it has been suggested that it may contribute to the formation of trimers (Grimes et al., 1995). Thus the effect of insertions into the VP7 top domain on VP7 trimer formation will also be investigated.

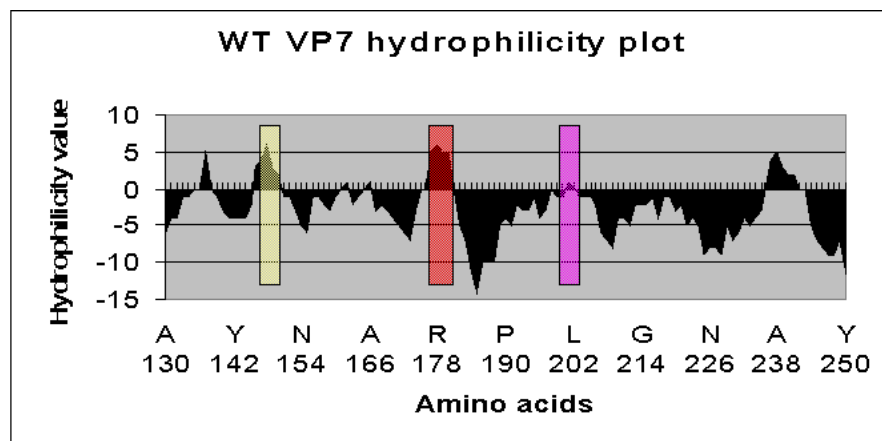


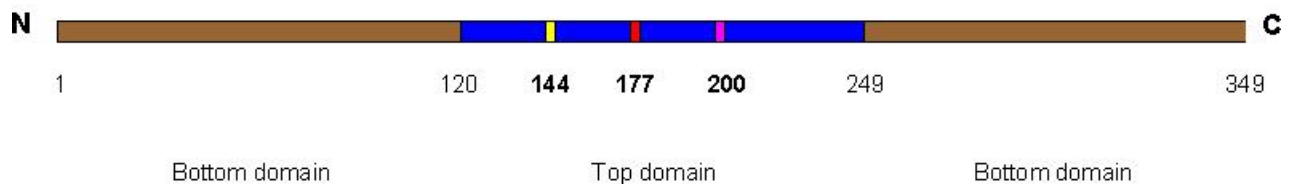
Fig 1.4: Hydrophilicity profile of wild-type AHSV-9 VP7 protein. Hydrophilicity was predicted according to the Hopp & Woods algorithm (Hopp & Woods, 1981, Hopp & Woods, 1983). Areas with positive values have a net hydrophilicity while areas with negative values have a net hydrophobicity. Shaded in yellow, red and pink are the 144-145, 177-178 and 200-201 sites, respectively, optimally located for the insertion of foreign peptides.

1.8 Antigenic peptides and marker proteins

In addition to the ability of the fusion protein to form trimers and its solubility, the ability of the inserted peptide to fold correctly within a particular site in the VP7 top domain and be presented in its optimal, native conformation on the surface of the VP7 trimer is another factor that will determine the effectiveness of the AHSV VP7 display system. To this end, a variety of well characterized, antigenic peptide sequences were inserted into the different insertion sites within the VP7 top domain. The peptides originated from the outer capsid protein VP1 of Foot-and-mouth disease virus (FMDV), the outer capsid protein VP2 of AHSV and the transmembrane protein gp41 of Human immunodeficiency virus (HIV-1). The humeral immune response elicited in small animals indicated whether the antigenic peptide was able to fold correctly and be presented in a conformation capable of eliciting insert-specific, neutralizing antibodies. The ability of an entire protein to fold correctly and retain its function when

inserted into the VP7 top domain was investigated by inserting the enhanced green fluorescence protein (e-GFP), 239 amino acids in length, into the three different sites within the top domain. The ratio of fluorescence emitted per unit protein would indicate in which of the 3 insertion sites the eGFP protein is able to fold and fluoresce most optimally. The effect of these insertions on the solubility of the VP7 protein and its ability to form trimers will also be investigated to determine if any factors that influence the above mentioned display criteria can be identified.

(A)



(B)

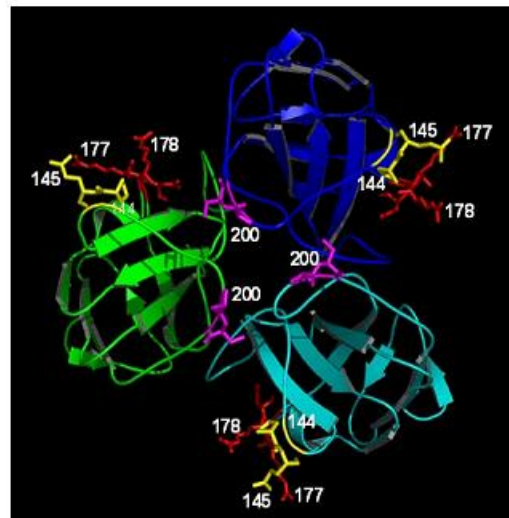


Fig 1.5: (A) Schematic representation of sites 144, 177 and 200 within the top domain of AHSV VP7 identified as being optimally located and solvent-accessible on the surface of the AHSV VP7 trimer molecule (Maree, 2000). (B) Computer generated model of the secondary structure of the top domain of an AHSV-VP7 trimer looking vertically onto the top of the trimer (Courtesy of Dr F. Maree). The spatial arrangement of the insertion sites 177-178 (Red), 144-145 (yellow) and 200-201 (pink) is indicated.

1.8.1 Foot-and-mouth disease virus (FMDV) VP1

Foot-and-mouth disease is a highly contagious, economically important disease of cloven-hoofed animals (Dunn & Donaldson, 1997, Knowles et al., 2001). Its etiological agent, Foot-and-mouth disease virus (FMDV), is a member of the genus *Aphthovirus* of the *Picornaviridae* family and is classified into seven distinct serotypes (O, A, C, SAT1, SAT-2, SAT-3 and Asia 1) and numerous subtypes (Doel, 2003). Safety concerns with the current inactivated FMD vaccine (Sutmoller & Casas Olascoaga, 2003), as well as difficulty in distinguishing between infected and vaccinated animals (Lu et al., 2007), has prompted the development of novel vaccine strategies, including subunit vaccines and synthetic peptides (Bittle et al., 1982, Brown, 1992, DiMarchi et al., 1986, Morgan & Moore, 1990, Su et al., 2007, van Lierop et al., 1995).

FMDV has an icosahedral non-enveloped viral capsid, composed of 60 copies of each of the four structural proteins, VP1, VP2, VP3 and VP4 (Sobrinho et al., 2001). Three of these proteins, VP1, VP2 and VP3, contribute to the formation of five known antigenic sites of type O₁ FMDV serotype (Aggarwal & Barnett, 2002, Crowther et al., 1993a, Kitson et al., 1990). The β G- β H loop (aa144-149, aa154) and carboxyl terminus (aa206-208) of the FMDV type O₁ VP1 protein contribute to linear antigenic site 1. The β G- β H loop of the SAT1 (aa135-151, aa156-169) and SAT2 (aa136-145, aa151-166) VP1 protein, as well as the carboxyl terminus of this protein, are also most likely involved in the formation of an antigenic site on the SAT-1/SAT-2 serotype virions (Maree et al., 2011). The importance of the VP1 protein in the antigenicity and immunogenicity of the FMDV was highlighted by the observation that protective, neutralizing antibodies were elicited in guinea pigs (Bittle et al., 1982) and cattle (DiMarchi et al., 1986, Morgan & Moore, 1990) following their immunization with peptides corresponding to amino acids 141-160 (Pfaff et al., 1982) and 200-213 (Strohmaier et al., 1982) of FMDV VP1. These peptides were also found to protect cattle and pigs against both homologous and heterotypic viral challenge with other FMDV serotypes (Doel et al., 1990). Subsequent studies showed that synthetic peptides composed of VP1 amino acids 134-158, which form the β G- β H loop, induced protection in mice and guinea pigs against virulent FMDV challenge (Zamorano et al., 1994, Zamorano et al., 1995) and a strong neutralizing humeral immune response was obtained in cattle (Zamorano et al., 1998). The cellular immune response induced in the natural host by these synthetic peptide-based vaccines was, however, lower than that elicited by the traditional inactivated vaccines and may be responsible for the limited protection observed against FMDV challenge in cattle (Borrego et al., 2006, Rodriguez et al., 2003, Taboga et al., 1997, van Lierop et al., 1995). In order to improve the cellular immune response the FMDV VP1 protein and a synthetic FMD multi-epitope peptide, were each individually fused to the *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) (Su et al., 2007). HSP70 is a chaperone which facilitates uptake of the bound peptide by antigen presentation cells (APC) and thus facilitates peptide presentation on major histocompatibility complex (MHC) molecules (Robert, 2003). The multi-epitope peptide consisted of five B cell regions, VP1aa141-160, VP1aa200-213, VP1aa135-167, VP1aa41-60, VP2aa70-77, VP2aa131-134, VP3aa55-59 and two T-cell regions VP1aa21-40 and VP1aa141-160. The humeral and cellular immune responses elicited by the fusion proteins were better than the responses elicited by

the antigen peptides alone indicating effective targeting of the APCs by the fused HSP70 protein. The cellular immune response induced by the fusion proteins was also more potent than that induced by the traditional inactivated vaccine. A recent study has indicated that a multi-epitope vaccine candidate, containing three repeats each of the antigenic sites VP1aa141-160 and VP1aa200-213 fused to a swine immunoglobulin G heavy-chain constant region (sclgG), was able to confer complete protection of swine against virulent homologous FMD viral challenge (Shao et al., 2011).

Unlike the FMDV A and O serotypes which occur globally (Samuel & Knowles, 2001), the South African Territories (SAT) serotypes 1, 2 and 3 are generally confined to sub-Saharan Africa (Bastos, 1998). In addition, the majority (41%) of FMD outbreaks in Africa from 2000 to 2010 were caused by serotype SAT2 viruses (Maree et al., 2011). In this study it will be determined whether the β G- β H loop (aa129-164) of the VP1 protein of the SAT2/ZIM/7/83 FMDV vaccine strain, a region that contributes to virus antigenicity (Crowther et al., 1993b, Maree et al., 2011), is able to elicit a humeral immune response when presented by the AHSV VP7 protein.

1.8.2 African horsesickness virus (AHSV) VP2

African horsesickness (AHS) is endemic to sub-Saharan Africa and poses a considerable risk to naïve populations of horses with a mortality rate of up to 95% (Coetzer & Guthrie, 2004). Due to its highly infectious nature, AHS, together with Bluetongue disease and Foot-and-mouth disease, are classified by the World Organization for Animal Health (OIE) as notifiable (Rodriguez-Sanchez et al., 2008). African horsesickness virus is grouped into nine different serotypes, AHSV-1 to AHSV-9 (Howell, 1962, McIntosh, 1958).

The outer capsid VP2 protein is the major target of the hosts neutralizing immune response against AHSV (Burrage et al., 1993, Martinez-Torrecuadrada & Casal, 1995, Martinez-Torrecuadrada et al., 1994, Martinez-Torrecuadrada et al., 1999, Vreede & Huisman, 1994). AHSV VP2 is capable of eliciting a protective neutralizing immune response (Burrage et al., 1993, Martinez-Torrecuadrada et al., 1994) and provides protection against homologous virulent viral challenge in horses (Roy et al., 1996b, Scanlen et al., 2002, Stone-Marschat et al., 1996). The use of AHSV VP2 as a subunit vaccine has, however, been restricted due to the tendency of this protein to aggregate when expressed in heterologous expression systems (Du Plessis et al., 1998, Venter et al., 2000). The inability of VP2 to fold into its native conformation, and subsequently expose the immunodominant epitopes required for the induction of neutralizing antibodies, has prompted the idea of using antigenically important VP2 peptides as immunogens.

In order to map the locations of antigenically important regions within the VP2 protein, (Martinez-Torrecuadrada & Casal, 1995, Martinez-Torrecuadrada et al., 1994) screened overlapping truncated protein fragments of AHSV-4 VP2 with neutralizing monoclonal antibodies and polyclonal neutralizing sera and identified a major antigenic region, located in the central part of the VP2 protein (aa200-413), containing several neutralization

sites. A peptide fragment comprising amino acids 253-413 was subsequently found to be able to elicit high titers of neutralizing antibodies in immunized mice (Martinez-Torrecuadrada & Casal, 1995). However, neutralizing antibodies in the sera of infected horses were not absorbed by this fragment indicating that this region is not immunodominant. Further investigation focused the AHSV-4 antigenic domain to amino acids 223-400 and peptide immunization of rabbits identified two neutralizing epitopes 'A' (aa321-339) and 'B' (aa377-400) (Martinez-Torrecuadrada et al., 2001). Utilizing phage display peptide libraries and monoclonal and polyclonal AHSV antibodies, (Bentley et al., 2000) identified two antigenic domains in AHSV-3 VP2 that were located within (aa228-283) or overlapped (aa393-432) the antigenic domain (aa223-400) identified by (Martinez-Torrecuadrada et al., 2001). A linear AHSV-9 VP2 epitope (aa398-418), overlapping the AHSV-4 antigenic region (aa 253-413) was also identified (Venter et al., 2000). However, expression of a number of these neutralization-specific peptides in insect cells rendered them insoluble and non-immunogenic (Venter et al., 2000). It will be investigated whether an AHSV neutralizing humeral immune response may be elicited by VP2 peptides presented by the AHSV VP7 protein.

1.8.3 Human immunodeficiency virus (HIV) gp41

Human immunodeficiency virus (HIV) is the causative agent of the acquired immunodeficiency syndrome or AIDS (Barre-Sinoussi et al., 1983, Gallo et al., 1984, Levy et al., 1984). HIV is a member of the *Lentivirus* genus in the family *Retroviridae*. HIV-1 strains fall into three divergent lineages, termed group M (Main), O (Outlier) and N (McCutchan, 2006). The major HIV pandemic is caused by HIV-1 group M strains which are divided into nine distinct genetic subtypes or clades A, B, C, D, F, G, H, J and K (McCutchan, 2000, Robertson et al., 2000). The global prevalence of Subtypes A, B, C and D is high compared to the other group M subtypes and viruses classified as group O, group N and HIV-2 (McCutchan, 2006). Subtype A is commonly found in East Africa, Central Asia and Eastern Europe while subtype B predominates in America, Western Europe and Australia. Subtype D is found principally in East Africa and, to a lesser extent, in West Africa. Subtype C is responsible for 50% of all HIV-1 infections worldwide and is concentrated in Southern and East Africa as well as in India (McCutchan, 2006).

A vaccine against HIV will have to stimulate both arms of the adaptive immune response, cellular as well as humeral immunity, if it is to provide a broad and long-lasting immunity against the diversity of HIV strains (Burton & Weiss, 2010). Although cell-mediated immunity is important in controlling viral infection, humeral immunity, in the form of broadly neutralizing antibodies in the blood and mucosal surfaces, will be the first step in protecting against HIV infection. The vulnerability of the virus to neutralization early in infection has been suggested by studies that show that productive HIV-1 infection is often mediated by one or only a few virions (McMichael et al., 2009). The development of an effective vaccine against HIV has been hampered by a number of mechanisms that the virus has evolved to elude immune control (Taylor et al., 2008). These include heavy glycosylation of the external glycoprotein, which protects the neutralizing epitopes (Sagar et al., 2006), the targeted infection of CD4⁺

immune cells, integration into the host cell genome and mutational escape from the immune system (Frost et al., 2005).

Structurally, HIV is an enveloped virion with 72 copies of the Env protein embedded in the lipid bilayer. Each Env is composed of a non-covalently associated, trimeric complex of gp120 and gp41 subunits (Chan et al., 1997, Weissenhorn et al., 1997). Exposed surface glycoproteins (gp120) are anchored to the virus via interactions with the transmembrane protein gp41. The native HIV-1 trimer is the sole target for neutralizing antibodies (Parren & Burton, 2001). Due to the ability of the HIV Env capsid protein's ability to evade the hosts immune system (Burton et al., 2004, Kwong et al., 2002, Wyatt & Sodroski, 1998), antibodies raised by vaccination with recombinant gp120 envelope protein from laboratory adapted HIV-1 isolates failed to neutralize heterologous challenge viruses in macaque and human populations (Belshe et al., 1998, Connor et al., 1998, Polacino et al., 1999). Indeed, most antibodies raised against the HIV-1 envelope glycoproteins are non-neutralizing (Mascola et al., 1996, Moore, 1995, Moore & Burton, 1999, Moore et al., 1995, Parren & Burton, 2001, Parren et al., 1997, Virgin & Walker, 2010) and fail to prevent HIV-1 infection, reduce viral loads or delay disease progression in human trials (Flynn et al., 2005, Pitisuttithum et al., 2006). This is most likely due to the high degree of HIV diversity especially within its Envelope protein (Env), as well as this proteins extensive glycosylation, sequestered receptor binding sites and resistance to conformational changes that allow for the exposure of functionally important domains (Burns & Desrosiers, 1994, Burton et al., 2004, Coffin, 1995, Kwong et al., 2002, Wyatt & Sodroski, 1998). It has, however, been found that broadly neutralizing antibodies (NABs) that target conserved regions on the Env trimer are elicited in a small subset of HIV-1 infected individuals over time (Burton, 1997, Geffin et al., 1998, Mascola & Montefiori, 2010, Stamatatos et al., 2009).

Passive neutralizing antibody transfer experiments in non-human primate models suggest that NABs may have a role for protection against HIV-1 *in vivo* (Ferrantelli et al., 2004b, Hessel et al., 2009a, Hessel et al., 2009b, Hessel et al., 2010, Mascola et al., 1999, Mascola et al., 2000, Ng et al., 2010). These include human monoclonal neutralizing antibodies b12 (Burton, 1997, Burton et al., 1994), 2G12 (Calarese et al., 2003, Sanders et al., 2002, Trkola et al., 1996, Wyatt et al., 1998) and 447-52D (Burton et al., 2004, Sharon et al., 2003) which all bind to the gp120 protein (Fig 1.7). Recent studies have identified additional anti-gp120 antibodies, PG9 and PG16 (Walker et al., 2009), HJ16 (Corti et al., 2010) and VRC01 (Li et al., 2007b), that are both potently and broadly neutralizing (Walker et al., 2010). Monoclonal antibodies 2F5 (Muster et al., 1993, Parker et al., 2001), 4E10 (Stiegler et al., 2001, Zwick et al., 2005, Zwick et al., 2001) and CL3 (Cotropia et al., 1996, Viveros et al., 2000), which bind to neighbouring linear epitopes on the membrane proximal external region (MPER) of gp41 and were originally isolated from individuals infected with HIV-1 group M subtype B, were found to neutralize not only to neutralize various primary HIV-1 subtype B isolates *in vitro* but also primary isolates of subtypes A, C, D, E and F (Kitabwalla et al., 2003, Stiegler et al., 2001, Trkola et al., 1995, Verrier et al., 2001, Xu et al., 2001, Zwick et al., 2001). In addition to being cross-subtype neutralizing, these antibodies were also found to have cross-group activity, neutralizing primary isolates of the phylogenetically distinct group O (outlier group) of HIV-1

(Ferrantelli et al., 2004a). These results indicate that these broadly neutralizing monoclonal antibodies recognize epitopes that are strongly conserved between subtypes, and even between groups, of HIV-1 and these epitopes would be ideal candidates for inclusion into an HIV-1 vaccine effective against a wide variety of different strains. The human monoclonal antibody (mAb) 2F5 is potently and broadly neutralising, with the ability to neutralise a variety of both laboratory and primary viral isolates of various subtypes (Conley et al., 1994, Muster et al., 1994, Muster et al., 1993, Purtscher et al., 1994, Trkola et al., 1995). Studies have found that 2F5, at acceptable concentrations, was able to neutralise 90% of the studied African, Asian, American and European primary isolates from subtypes A, B and E (Purtscher et al., 1996, Purtscher et al., 1994). In a complementary study, 2F5 was one of three antibodies capable of neutralising a majority of typical U.S. primary isolates (D'Souza et al., 1997). In addition, chimpanzees infused with 2F5 were able to delay the establishment of infection and viremia after inoculation with a primary isolate (Conley et al., 1996) and, more recently, this antibody was also found to completely protect against mucosal SHIV challenge (Hessell et al., 2010). Although mAb 2F5 has great protective capabilities, its occurrence in infected individuals is rare (Burton, 1997). At least 80% of HIV-1 infected children do not elicit the antibody (Geffin et al., 1998) and it has never been raised by a vaccine candidate (Moore & Trkola, 1997). The 2F5 antibody binds to the ELDKWA epitope, primarily at the core amino acid sequence LDKW (Muster et al., 1994, Muster et al., 1993, Ofek et al., 2004, Purtscher et al., 1996, Purtscher et al., 1994, Sattentau et al., 1995). This highly conserved subtype B epitope is located adjacent to the C-terminal of gp41 (aa669-674) (Dong et al., 2001, Muster et al., 1993). Another promising protective epitope is RILAVEYLKD situated on the N-terminal of gp41 (aa586-596) (Chen & Dierich, 1998, Dong et al., 2001, Dorn et al., 2000). This highly conserved epitope is responsible for the binding of gp41 to the potential receptor protein P45 (Xiao et al., 2000a). A similar epitope, SIV gp32, was found to protect macaques from SIV infection (Shafferman et al., 1991).

Human mAb CL3 is able to bind to and neutralize infection of chronic T cell lines and human peripheral blood mononuclear cells (PBMCs) by laboratory-adapted HIV-1 (Cotropia et al., 1996, Viveros et al., 2000). In addition, this antibody is also able to neutralize infection of PBMC by clinical HIV-1 isolates from the B, E, and F subtypes (Kliks et al., 1994). CL3 binds to the amino acid sequence GCSGKLICTT within the ectodomain of gp41 (aa602-611). The two cysteine residues within this sequence form an intrachain disulphide bridge and, hence, a loop (Goudsmit et al., 1990). This loop may be important for the folding of gp41 during fusion of the virus with the target cell (Chan & Kim, 1998). Further characterisation reveals that this is a linear epitope, with the amino acids LIC crucial for antibody binding (Viveros et al., 2000). CL3 was also found to be able to bind to oligomeric glycoproteins, indicating that the epitope within the cluster I region is presented efficiently on HIV-1. Although these authors were unable to raise neutralising epitope-specific immune response using a peptide-based vaccine in rabbits, this does not mean that the epitope cannot be presented in a context capable of eliciting neutralising antibody. Indeed, recent studies show that neutralizing antibodies, induced by HIV envelope vaccines, can confer complete protection against homologous SHIV challenge in macaques (Barnett et al., 2008, Bogers et al., 2008, Pal et al., 2006). Thus if appropriate immunogens, adequately presenting the conserved

epitopes described above, can be designed protective humeral immunity against HIV-1 may be elicited. Peptide-based vaccines, containing the above-mentioned epitopes, have successfully elicited high titres of epitope-specific antibodies and have been discussed in detail in section 1.6 (Peptide-based vaccines). However, the conjugation of these peptides to carrier protein such as Bovine serum albumin is expensive (Chackerian, 2007) and it is also difficult, in most cases, for peptide-based vaccines to elicit an effective cellular immune response (Liu et al., 2003). The presentation of HIV epitopes on VP7 trimers may not only inherently increase the number of epitope copies being presented to the immune system, thereby increasing the immunogenicity of the epitope (Jegerlehner et al., 2002, Liu & Chen, 2005), but also provide a means of presenting HIV-1 CTL or Th cell epitopes and inducing a cellular immune response.

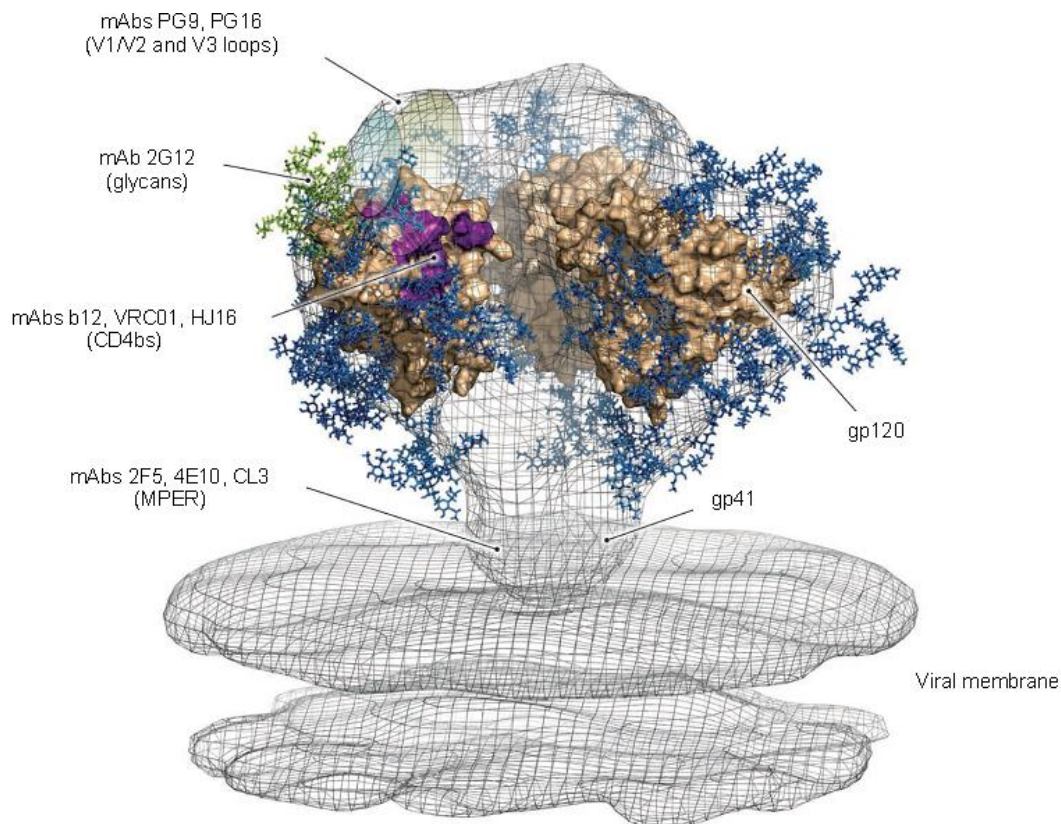


Fig 1.7: Computer model of the HIV-1 envelope heterotrimer, containing viral glycoproteins (gp120)₃(gp41)₃, generated from combined cryoelectron tomographic and crystallographic data (Burton, 2010, Walker, 2010b). The approximate locations of the epitopes, targeted by the existing broadly neutralizing monoclonal antibodies (mAbs), on the gp120 and gp41 proteins are indicated. The gp120 core structure (tan) is fitted in the electron density map (gray) of the heterotrimer. The membrane proximal external region (MPER) and the viral membrane are also shown.

1.8.4 Enhanced green fluorescent protein (eGFP)

Green fluorescent protein (GFP) is the prototypical member of a diverse family of fluorescent and non-fluorescent proteins expressed by several marine organisms. It was first isolated from the jellyfish *Aequorea victoria* in 1962 (Shimomura, 2009, Shimomura et al., 1962). It, however, remained in relative obscurity for another thirty years before the *gfp* gene was cloned (Prasher et al., 1992). Its subsequent expression in heterologous systems, such as *Escherichia coli* and *Caenorhabditis elegans*, gave rise to a fully functional fluorescent GFP protein (Chalfie, 2009, Chalfie et al., 1994) and this indicated that GFP forms spontaneously and does not require any additional enzymes or co-factors. Advanced variants of *A. victoria* GFP were developed by mutagenesis, with emission peaks ranging from Blue (BFP) to yellow (YFP) (Shaner et al., 2007, Tsien, 1998). Homologs of GFP were also discovered in anthozoa animals increasing the number of fluorescent proteins available (Lukyanov et al., 2010, Matz et al., 1999, Pakhomov & Martynov, 2008, Verkhusha & Lukyanov, 2004, Wiedenmann et al., 2000). The spontaneous formation of GFP, and its variants, makes them ideal genetically encoded fluorescent markers that can be fused to proteins of interest and used for the visualization of protein expression, localization, translocation, interactions and degradation within living systems in real time (Chudakov et al., 2010, Nienhaus & Wiedenmann, 2009, Wiedenmann et al., 2009). Not only does GFP retain its fluorescence in the context of genetically engineered fusion proteins, it also does not generally affect the function and/or localization of its fusion partner within the cellular environment (Zimmer, 2002). The expression of a fluorescent fusion protein provides an advantage over other visualization techniques as it obliterates the need to first conjugate the purified protein of interest to a fluorescent or radioactive dye prior to screening (Chalfie et al., 1994, Stearns, 1995, Zhang et al., 1996). In addition to the qualitative assays, such as protein translocation and localization (March et al., 2003), quantitative assays that indicate levels of protein expression (Cha et al., 2005, Hyung et al., 2005, Shin et al., 2003) or enzymatic activity (Li et al., 2007a) may also be performed with GFP fusion proteins. The fluorescence emitted by the GFP fusion protein is a relative value that can be correlated to the quantity of protein being expressed.

Wild-type GFP from *A. Victoria* is a 27 kDa protein composed of 238 amino acids. Following translation the proteins folds into an 11-stranded β -barrel structure with a single α -helix passing through the interior of the barrel (Fig 1.6) (Tsien, 1998, Zimmer, 2002). The fluorescent chromophore, 4-(*p*-hydroxy-benzylidene)-5-imidazolinone, interrupts the helix and is held rigidly close to the geometric center of the barrel-shaped protein, protected from the bulk solvent. The fluorescing chromophore is generated auto catalytically from the tripeptide Ser65-Tyr66-Gly67 when the GFP protein folds into its near native conformation (Barondeau et al., 2003, Craggs, 2009). Whilst Gly67 is crucial for chromophore self-synthesis, the remaining amino acids may be substituted and this has led to the development of many of the GFP variants utilized today (Heim et al., 1994, Shaner et al., 2004). The N- and C-terminals of the protein are situated proximal to one another at the base of the β -barrel (Yang et al., 1996) so that the GFP-like protein can be fused to either the N-terminus or C-terminus of the protein of interest (Kratz et al., 1999, Miyawaki et al., 2003). In many cases fusion of the GFP-

like protein to one of the termini of the target protein is preferable due to the involvement of the other terminus in specific interactions or its role in determining the proteins localization (Chudakov et al., 2010). It is important that the literature be investigated thoroughly in order to determine which terminus is most suited for GFP-like protein fusion. In some cases, the performance of a fusion protein may be improved by inserting a flexible Gly-rich linker of several amino acids between the GFP-like protein and the protein of interest, to prevent potential steric conflicts (Nagai & Miyawaki, 2004). Although insertion of the GFP-like protein into the amino acid sequence of the target protein is usually avoided as it may affect the correct folding of the target protein, it is sometimes necessary due to the importance of both termini of a target protein or other considerations. Such insertions have been successfully achieved (Baehler et al., 2002, Moradpour et al., 2004, Rocheleau et al., 2003, Senejani & Gogarten, 2007). The insertion of peptides into hydrophilic loops, exposed on the surface of virion capsids, may be tolerated provided that these insertions do not interfere with the formation of the major secondary structures of the protein. The insertion of the entire green fluorescent protein (GFP), flanked by flexible linkers, into a short loop, containing the surface exposed c/eI epitope (Conway et al., 1998), on the hepatitis B capsid protein (HBcAg) not only resulted in the production of fluorescent particles, which indicated correct folding of both the capsid HBcAg protein and the GFP protein into their native three dimensional structures, but also resulted in the exposure of GFP on the surface of the HBcAg particle and the induction of a strong humeral immune response against the GFP protein (Kratz et al., 1999).



Fig 1.6: Structure of green fluorescent protein (GFP) monomer (Nienhaus & Wiedenmann, 2009). The protein backbone is depicted as ribbons while the chromophore is represented by van der Waals spheres.

In addition to enabling the visualization of target protein expression and localization, fluorescent protein markers are also used to determine whether the protein targets that they are fused to are correctly folded, functional and stable (Shaner et al., 2007, VanEngelenburg & Palmer, 2008, Yen et al., 2008). When employing fluorescent proteins for this function it is assumed, firstly, that they fold autonomously into their native fluorescent state when

expressed as part of a fusion protein and, secondly, that a quenching of the fluorescence emitted reflects unfolding/misfolding of the protein leading to the chromophore being exposed to the bulk solvent (Hsu et al., 2009). Thus the intrinsic fluorescence of these marker proteins is frequently used to assess their folding state as well as the folding state of the protein targets to which they are fused (Giepmans et al., 2006). The GFP folding reporter technology was developed to screen for protein variants, synthesized by random mutagenesis, which exhibited improved folding and solubility characteristics (Waldo et al., 1999). One of the factors influencing the solubility of a protein is protein folding. Test proteins were expressed as an N-terminal fusion with GFP and it was demonstrated that the fluorescence of these GFP-fusion proteins was related to the correct folding of the upstream test protein expressed individually. It was hypothesized that a poorly folded test protein interferes with the correct folding of the fused GFP marker protein resulting in a quenching of the fluorescence emitted. Directed protein evolution using cycles of mutagenesis and selection via GFP folding interference has been used to engineer soluble variants of a number of misfolded, insoluble proteins from several organisms (Graziano et al., 2008, Kim & Hecht, 2005, Kim & Hecht, 2006, Olson & Roberts, 2007, Pedelacq et al., 2002, van den Berg et al., 2006, Wurth et al., 2002, Yang et al., 2003). Correctly folded, soluble viral surface protein fragments, from the surface glycoproteins of SARS-CoV and HIV-1, were also identified using GFP as a folding reporter and could be used as vaccine candidates (Lin et al., 2009). As truncated, soluble correctly-folded polypeptides may also result in the correct folding of the GFP protein, false positives may result when making use of this technology (Kawasaki & Inagaki, 2001). In order to circumvent this problem, the split-GFP solubility reporter technology was recently developed (Cabantous et al., 2005a, Cabantous et al., 2008, Cabantous et al., 2005b, Listwan et al., 2009). Misfolding by the test protein would be expected to interfere with the folding and assembly of the two GFP domains and the reconstitution of the active fluorophore. The split GFP complementation system has recently been used to quantitatively measure the tau protein, known to be involved in Alzheimers disease, in living cells (Chun et al., 2011).

Enhanced green fluorescent protein (eGFP) is a variant of GFP which was synthesized by mutating amino acid 65, forming part of the chromophore, from a serine to a threonine (Heim et al., 1995). The mutation of amino acid 64, proximal to the chromophore, from a phenylalanine to a leucine, improved the level of fluorescence at 37°C (Cormack et al., 1996). Additional mutations have also been described (Tsien, 1998). eGFP is commercially available and fluoresces thirty-five times brighter than GFP with an excitation/emission maxima 480/505nm. The structure of this 239 amino acid protein was initially obtained in 1996 prior to the introduction of the F64L mutation (Ormo et al., 1996), but the structure of eGFP with all its current mutations has only recently been resolved (Royant & Noirclerc-Savoye, 2011). In this study the eGFP protein will be inserted into, amongst other positions, a hydrophilic loop (aa168-176), containing the surface-exposed RGD motif (Basak et al., 1996), within the top domain of AHSV VP7 and its fluorescence will be used to assess the folding and solubility of the resulting VP7-eGFP fusion protein. The formation of fluorescing VP7-eGFP trimers would indicate the correct folding of the VP7 protein as well as the inserted eGFP protein. This would support the development of AHSV VP7 as an antigen presentation system for the delivery of even large proteins to the immune system.

1.9 Aims

The aim of this study is to determine whether the major capsid protein VP7 of AHSV may be used as a general purpose antigen delivery and display system. The effective display of antigenic peptides by VP7 would depend on the ability of the VP7 fusion protein to form trimers, the solubility of these trimers as well as the ability of the inserted peptide to be presented in its native immunogenic state on the surface of the VP7 trimer molecule. The more specific questions addressed in this study are:

- The effect of inserting 6-mer peptides into different positions within the AHSV-9 VP7 top domain, during VP7 vector construction, on protein solubility, particle morphology and trimer formation
- The effect of substituting a hydrophobic leucine residue, in position 345 of the VP7 bottom domain, with the BTV VP7 equivalent, a hydrophilic arginine residue, on protein solubility and trimer formation
- The effect of inserting larger, foreign peptides, derived from the outer capsid proteins VP1 of FMDV and VP2 of AHSV and the envelope protein gp41 of HIV-1, into different sites within the VP7 top domain on protein solubility and trimer formation. The effect of inserting an entire protein, the enhanced green fluorescent protein (eGFP), 239 amino acids in size, into the VP7 top domain is also investigated.
- The ability of the inserted foreign peptides, and the eGFP protein, to fold correctly when inserted into the VP7 vectors and the effect on their immunogenic or fluorescing functions when displayed on the surface of VP7 trimers
- The VP7 fusion protein fraction, particulate or soluble, responsible for eliciting the superior humeral immune response
- The effect of various solubilization strategies, including treatment with solubilizing agents Na⁺N-Lauroylsarcosine (sarkosyl) and the amino acid L-arginine, on VP7 fusion protein solubility
- The effect of sarkosyl and L-arginine on the humeral immune response elicited by the solubilized VP7 fusion protein
- The use of the insoluble VP7-HIV fusion particles in HIV serum characterization

Chapter 2

The effect of VP7 modifications on AHSV VP7 protein solubility, trimer formation and the induction of an immune response

2.1 Introduction

Immunologically important epitopes may be displayed on the surface of virus-like particles (VLPs) in order to elicit an immune response (Pumpens, 2008). Such a particle display strategy has been explored for Bluetongue virus (BTV), the prototype member of the *Orbivirus* genus. When co-expressed in insect cells, the BTV VP7 and VP3 proteins spontaneously assemble into core-like particles (CLPs) that resemble the viral core particles (French & Roy, 1990). Various foreign epitopes inserted into these proteins have been presented on the surface of such chimeric BTV CLPs (Adler et al., 1998, Belyaev & Roy, 1992, Tanaka et al., 1995). However, this CLP strategy is limited to only those chimeric proteins that are able to assemble into stable CLPs (Tanaka et al., 1995). This strategy is also not viable with African horsesickness virus (AHSV), a closely related orbivirus, as co-expression of the AHSV VP7 and VP3 proteins within insect cells results in a low yield of CLPs, with a number of crystal-like structures being observed instead (Maree et al., 1998).

AHSV VP7 is a hydrophobic protein (Roy et al., 1991). When expressed in AHSV-infected cells, or recombinant baculovirus infected insect cells, AHSV VP7 forms hexagonal, disc-shaped crystal-like particles, approximately 6µm in diameter (Burroughs et al., 1994, Chuma et al., 1992). The highly ordered and symmetric structure of these VP7 crystal-like particles was found to be similar to that of the VP7 capsomers on the outer surface of the AHSV core (Burroughs et al., 1994) and these particles were subsequently confirmed to be composed of VP7 trimers (Monastyrskaya et al., 1997). As VP7 crystal-like particles protect mice against virulent AHSV challenge in a subunit vaccine strategy (Wade-Evans et al., 1997), it was suggested that antigenic peptides may be effectively displayed to the immune system by VP7 trimers assembled into such particles. AHSV VP7 vector proteins were constructed for the display of such peptides by our research group (Fig 2.1; Table 2.2). The insertion of amino acids KLSRVD or P₁ downstream of amino acids 144, 177 and 200 of the AHSV-9 VP7 protein generated VP7 vector proteins VP7-144(P₁), VP7-177(P₁) and VP7-200(P₁), respectively. The range of cloning sites was complemented by vector VP7-144(P₂), in which peptide PGEFLE or P₂ was inserted downstream of VP7 amino acid position 144. For the simultaneous presentation of multiple epitopes, vectors VP7-144(P₂)-200(P₁) and VP7-144(P₂)-177(P₃)-200(P₁) were also constructed. It was not known how these 6-mer peptide insertions within different sites of the top domain of AHSV VP7 would affect this proteins solubility and ability to form trimers. These two criteria are important in the development of AHSV VP7 protein as an antigen presentation system. In addition to the modifications within the top domain of VP7, the effect of

substituting a hydrophobic leucine residue in position 345 of AHSV-9 VP7, close to the C-terminal and within helix 9 of the bottom domain of this protein (Fig1.2B), with the equivalent hydrophilic arginine residue of BTV VP7 (Monastyrskaya et al., 1997) on VP7 solubility and trimer formation must also be determined. Preliminary results indicate that this modification may increase VP7 protein solubility (Meyer, 2002).

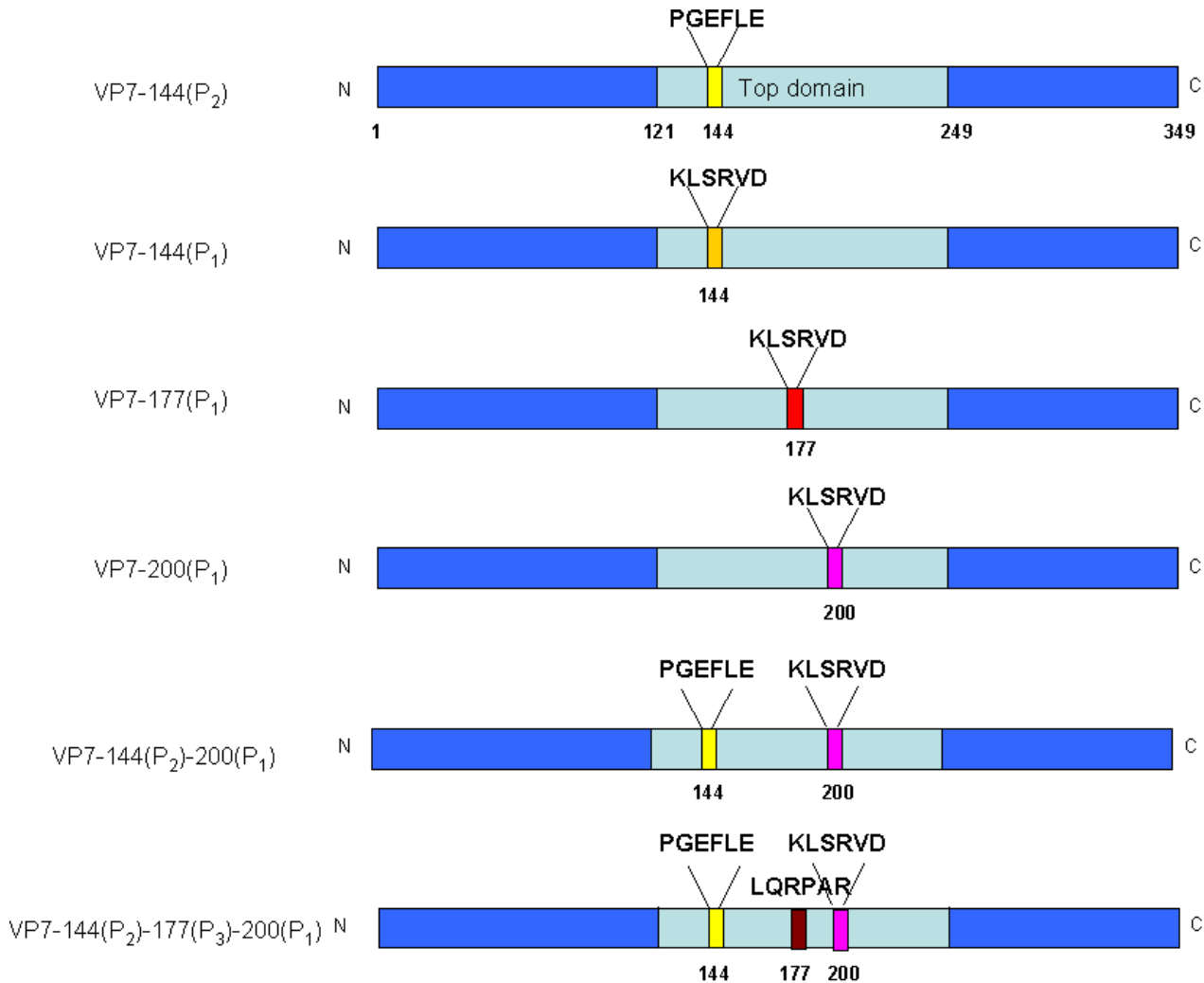


Fig 2.1: A schematic representation of the six different VP7 vector proteins analyzed in this study. The 6-mer peptides P₁, P₂ and P₃ were inserted downstream of amino acids 144, 177 and 200 in the AHSV VP7 top domain (amino acids 121-249) as indicated.

The Bac-to-Bac[®] baculovirus expression system (Luckow et al., 1993) was used for the expression of the VP7 proteins in this study. The VP7 vector, the C-terminal modified VP7 and VP7-FMDV fusion proteins were expressed in recombinant baculovirus-infected *Spodoptera frugiperda* (Sf9) insect cells. The Bac-to-Bac[®] baculovirus expression system is based on site-specific transposition of an expression cassette into a

baculovirus shuttle vector (bacmid), propagated in *E.coli* (Luckow et al., 1993). This strategy circumvents the long process of homologous recombination between a recombinant transfer vector and the parent *Autographa californica* nuclear polyhedrosis viral (AcNPV) genome and improves the ease with which recombinant baculoviruses are generated. In addition to increasing the frequency of recombination, this system also eliminates the need for repeated rounds of plaque purification in order to purify recombinant virus from residual wild type (wt) virus. The advantages of utilizing baculovirus-expressed proteins for vaccination regimes thus include the generation of large quantities of post-translationally modified protein, lack of protein contamination with infectious virus and easy purification with a one step ultracentrifugation process.

The solubility of the WT-VP7 and modified VP7 proteins will be determined by sucrose density gradient sedimentation analysis in this study. Step gradients will be prepared by layering several sucrose solutions of different concentrations in an ultracentrifuge tube. Samples will be layered on top of the gradients in order to reduce contamination by cell debris, which is reported to remain within the top sucrose layer following separation (Pertoft, 2000). The centrifugation conditions used would allow for the separation of the soluble and insoluble VP7 fractions according to their sedimentation co-efficient and buoyant density. Solubility analysis under the same experimental conditions would allow for a direct comparison of the solubility of the different modified VP7 proteins.

The ability of the WT-VP7 and modified VP7 proteins to form trimers will be assessed in one of two ways, either via low density sucrose sedimentation analysis or by means of a modified SDS-polyacrylamide gel electrophoresis strategy (Monastyrskaya et al., 1997). Glycerol gradient sedimentation of soluble VP7 oligomers with size marker proteins has previously been used to determine the size of the soluble VP7 oligomer (Limn & Roy, 2003, Limn et al., 2000). Similarly in this study, low density sucrose gradient sedimentation analysis will be used to assess the size of modified VP7 oligomer. Trimerization of modified VP7 proteins that cannot be assessed via the sedimentation protocol will be determined by making use of a modified SDS-polyacrylamide gel electrophoresis protocol, described previously (Monastyrskaya et al., 1997). This protocol is based on evidence that WT AHSV VP7 trimer molecules are stable in the presence of SDS at room temperature. The ability of modified VP7 protein to form trimers will thus be assessed by subjecting the protein to electrophoresis through a SDS polyacrylamide gel without prior denaturation at 95°C.

In addition to their effect on VP7 solubility and trimer formation, the effect of VP7 modifications on particle morphology will also be investigated. WT VP7 AHSV has previously been observed under the electron microscope to form flat hexagonal crystal-like particles (6µm) (Burroughs et al., 1994, Chuma et al., 1992). Whether the modified VP7 proteins are still capable of forming trimers that assemble into these crystal-like structures will be determined by viewing the particulate fraction of the WT and modified VP7 proteins with the Scanning Electron Microscope (SEM).

As AHSV VP7 trimers are proposed as an antigenic peptide display system, it is important not only to assess the solubility of the VP7 fusion protein and its ability to form trimers but also the ability of the inserted peptide to fold correctly and be presented in its native, immunogenic conformation on the surface of the VP7 trimer. The peptide selected for insertion into the top domain of VP7 was a well-characterized Foot-and-mouth disease viral (FMDV) peptide derived from the VP1 outer capsid protein and containing a cluster of highly conserved B-cell and T-cell epitopes (Aggarwal & Barnett, 2002, Pfaff et al., 1982, Robert, 2003). This 36-mer peptide was inserted into the VP7-177(P₁) vector protein (Dr Fick, personal communication). The effect of this insertion on VP7 solubility and trimer formation will be investigated in this study as well as the ability of this peptide to elicit a FMDV neutralizing humeral immune response in guinea pigs.

Should the chimeric VP7-FMDV fusion protein have an insoluble (particulate) as well as a soluble fraction, these will be inoculated into the test animals to determine which is capable of eliciting the superior humeral immune response. This would give an indication of the importance of the VP7 fusion protein solubility in the induction of a humeral immune response. In addition, antigen will also be injected in the presence of Complete Freund's adjuvant (CFA) or Incomplete Freund's adjuvant (IFA). Incomplete Freund's adjuvant, lacking the *Mycobacterium tuberculosis* component, has previously been used to minimize the adverse localized reaction of animals to the complete form of the adjuvant (Oscherwitz et al., 2006). If the humeral immune response elicited against VP7-177(P₁)-FMDV_{epi} with IFA is equivalent to that elicited by VP7-177(P₁)-FMDV_{epi} with CFA, the former may be used as a substitute for the complete form of the adjuvant in future animal trials. The antisera from the inoculated guinea pigs will be tested for insert-specific antibodies using an indirect ELISA (Enzyme-Linked Immunosorbent Assay) protocol and neutralizing antibodies using a FMDV neutralizing assay.

The indirect ELISA is a sensitive, accurate and reliable technique used for the detection (qualitative) and measurement (quantitative) of specific antibody in a test sample. The initial step of this technique involves coating the well of a polystyrene plate with antigen by adsorption. Test antiserum is added and allowed to incubate. If any antibodies in the test antiserum have bound to the immobilized antigen, their presence is detected via an enzyme-linked anti-immunoglobulin. Enzyme substrate is then added to react with the enzyme. Enzyme activity causes a colour change in the substrate solution that can be monitored visually or measured by means of a spectrophotometer. The rate of enzyme activity is directly proportional to the quantity of enzyme-linked antibody present which, in turn, is proportional to the amount of specific antibody present in the test sample. The protocol used in this study to detect FMDV-specific antibodies in guinea pig sera is a modified indirect ELISA assay as an anti-FMDV antibody was first used to coat the wells of the polystyrene plate prior to the addition of the purified FMDV virus (antigen), followed subsequently by the addition of the test antiserum and finally the addition of the enzyme-linked anti-immunoglobulin.

Neutralizing antibodies are those specific antibodies that cause loss of viral infectivity by binding to the virus at sites that are required for adsorption and entry into the host cell. The presence and titer of neutralizing antibodies in the sera of inoculated animals will be determined by means of a virus neutralization assay, as

described in the OIE Manual of Standards (2009). Prior to the FMDV neutralization test, the titer of the FMDV SAT2/ZIM7/83 virus will be determined by preparing serial tenfold dilutions of this isolate and inoculating these dilutions into susceptible IB-RS-2 cells in culture (Rweyemamu et al., 1978). The virus endpoint titer is the reciprocal of the highest dilution of virus that infects 50% of the host system, for example 50% of cell cultures develop CPE. This endpoint dilution contains one 50% tissue culture infecting dose (TCID₅₀) per unit volume. The concentration of virus used in the neutralization test will be 100 TCID₅₀ per unit volume. Serial twofold dilutions of serum will be prepared and mixed with an equal volume containing 100 TCID₅₀ of FMDV SAT2/ZIM7/83 virus. The virus and serum mixtures will be incubated for 1 hour at 37°C and then inoculated into susceptible IB-RS-2 cells in culture. The endpoint titration contains one antibody unit and is the reciprocal of the highest dilution of the antiserum protecting against the virus.

2.2 Materials and Methods

2.2.1 Insect cell culture

Spodoptera frugiperda (Sf9) cells were obtained from the NERC Institute of Virology, Mansfield Road, Oxford, UK. The cell culture medium consisted of TC100 Medium, supplemented with 10% (v/v) foetal calf serum (FCS), 1.2 × antibiotic-antimycotic solution, all supplied by Highveld Biological, and 0.08% Pluronic F-68 (Sigma Life Science). Sf9 cells were maintained as suspension cultures in spinner flasks (Pyrex) at 27°C with rotational shaking at 114 rpm in a Labcon rotational shaker. The cell density of the suspension cultures was determined by making use of a haemocytometer (Neubauer), with 0.4% trypan blue (Sigma Life Science) in 1 × phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·2H₂O, 1.4 mM KH₂PO₄, pH 7.3) serving to determine cell viability. Once cell cultures had attained a density of 2-2.4 × 10⁶ cells/ml, they were sub cultured to a lower density of 0.2-0.5 × 10⁶ cells/ml in a minimum volume of 50 ml.

2.2.2 Generation of recombinant baculovirus

Recombinant baculoviruses were generated by the Bac-to-Bac[®] baculovirus expression system (Invitrogen). *Spodoptera frugiperda* (Sf9) cells were seeded in a 6-well plate (Nunc[™]) at a density of 1 × 10⁶ cells/ 35 mm well. The cells were allowed to attach at 27°C for at least 1 hr. Cells were transfected with bacmid DNA using Cellfectin (Invitrogen), a cationic liposome reagent optimised for the transfection of insect cells. Five microlitres of newly isolated bacmid DNA was added to 100 µl Grace's Insect medium, not containing any supplements. A second solution containing 6 µl Cellfectin reagent in 100 µl Grace's Insect medium, without supplements, was also prepared. The two solutions were combined, mixed gently and incubated for 45 min at room temperature without any agitation. This step allows for the formation of lipid-DNA complexes. The attached Sf9 cells were washed with 2 ml Grace's Insect Medium (No supplements). Eight hundred µl Grace's Insect medium was added to each tube containing lipid-DNA complexes and mixed gently. The wash medium was removed and 1 ml of the

diluted DNA-lipid complexes placed on the cells in each well. Cells were incubated at 27°C for 5 hr. The transfection mixtures were then removed and replaced with 2 ml cell culture medium, containing FCS as well as antibiotics. The cells were incubated at 27°C for 96 hr, after which the virus-containing supernatant was harvested and stored at 4°C. Pure recombinant virus stocks were prepared from single plaques using established procedures. A recombinant plaque-purified baculovirus, capable of expressing wild type (WT) AHSV-9 VP7 in Sf9 cells (Maree et al., 1998) was available at the beginning of this investigation. Also available was a baculovirus recombinant that expressed the BTV-10 VP7 gene, generated by Dr Wilma Fick from a BTV-10 virus stock originally obtained from Onderstepoort Veterinary Institute (OVI).

2.2.3 Modified AHSV-9 VP7 constructs

All the constructs were designed for expression by baculovirus recombinants as described in the Bac-to-Bac[®] baculovirus expression system manual (Invitrogen). Briefly, plasmid vector pFastBac1-VP7, constructed to generate a recombinant baculovirus expressing the AHSV-9 VP7 gene (Maree et al., 1998), was used as template in the cloning strategy to generate the vector proteins VP7-144(P₁), VP7-177(P₁), VP7-200(P₁) and VP7-144(P₂). In order to construct vector proteins VP7-144(P₁), VP7-177(P₁), VP7-200(P₁), the VP7 vector gene was amplified as two fragments from the pFastBac1-VP7 DNA template using the primers detailed in Table 2.1. For example, in the construction of VP7-144(P₁), internal oligonucleotide primer O1F was used in combination with VP7 external primer O10R to amplify the 3' end fragment. Primers O2R and O9F were similarly used to amplify the 5'-end fragment. The resulting VP7 amplicons of the 5' and 3' end gene fragments were first cloned into the pMosBlue-T vector (GE Healthcare) and then re-cloned into the *Bam*HI/*Xba*I and *Xba*I/*Kpn*I restriction enzyme (RE) sites of plasmid pFastBac1 (Invitrogen), respectively, following the removal of the additional *Hind*III site present in the pFastbac1 multiple cloning site (Dr Q. Meyer, personal communication). VP7-177(P₁) and VP7-200(P₁) were constructed using the same strategy (Maree, 2000) resulting in the insertion of an 18 nucleotide DNA fragment specifying RE sites *Hind*III, *Xba*I and *Sal*I downstream of the codons encoding amino acids 144, 177 and 200 of the AHSV-9 VP7 gene. A fourth cloning vector, VP7-144(P₂), was generated by inserting a DNA fragment that specifies RE sites *Sma*I, *Eco*RI and *Xho*I in the VP7 gene (Riley, 2003). In addition, the double vector VP7-144(P₂)-200(P₁) was generated by inserting a DNA fragment that specifies RE sites *Sma*I, *Eco*RI and *Xho*I into the VP7-200(P₁) gene (Riley, 2003) while triple vector VP7-144(P₂)-177(P₃)-200(P₁) was constructed by inserting a DNA fragment that specifies RE sites *Pst*I, *Stu*I, *Bss*HI into the VP7-144(P₂)-200(P₁) gene (Van Rensburg, 2004).

The high fidelity TaKaRa Ex Taq[™] PCR kit (TAKARA BIO INC.) was used to perform all the PCR amplifications. The 50µl PCR reactions were set up according to the manufacturer's instructions using a final concentration of 0.5µM for each primer and the following cycling conditions: 1 cycle of 94°C for 3min, followed by 30 cycles of 94°C for 20 sec, 55°C for 30 sec and 72°C for 45 sec followed by 1 cycle of 72°C for 15min. The oligonucleotide

primer sequences used are provided in Table 2.1 with the restriction enzyme sites highlighted in bold. All amplicons and constructs were verified by chain termination sequencing (Applied biosystems).

To introduce a site specific leucine to arginine substitution mutation at positions 345 and 351 in WT-VP7 and VP7-177(P₁), respectively, an inverse PCR strategy was used using oligonucleotide primers O21F and O22R (Meyer, 2002). Plasmids pFastBac1-VP7 and pFastBac1-VP7-177(P₁) were used as templates. The strategy was designed to create a *Sma*I site at the target nucleotide position which resulted in the required codon change after *Sma*I digestion and self ligation of the PCR amplicon. PCR cycling conditions were as follows: 1 cycle of 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 58°C for 30 sec, 72°C for 6 min and 1 cycle of 72°C for 15 min.

For the construction of VP7-177(P₁)-FMDVepi fusion protein, a sequence, encoding a cluster of B-cell epitopes (amino acids 129-164) located on the 1D gene of FMDV, was amplified using a plasmid containing a part of the genome of FMDV vaccine strain SAT2/ZIM/7/83 as template (Dr Fick, personal communication). The resulting PCR amplicon was cloned into vector VP7-177(P₁) utilizing the *Hind*III and *Sal*I sites.

All the modified AHSV-9 VP7 proteins investigated in this study are listed in Table 2.2. This table is a reference for the specific modifications introduced into VP7 and also details the authors responsible for these modifications.

2.2.4 Expression and harvesting of VP7 protein

S. frugiperda cells in 75cm³ tissue cultures flasks (Nunc) (1 x 10⁷ cells per flask) were infected with recombinant baculoviruses at an m.o.i of 5-10 pfu/cell. The expressed protein was harvested 72 hours post infection by dislodging the cells from the surface of the flask and pelleting them via centrifugation at 829 xg (2000rpm) for 5 min in a Beckman Coulter Avanti J-E centrifuge with a JS-5.3 rotor. The pellet was resuspended in 800µl lysis buffer (0.01M STE with 0.5% Nonidet[®]-P40 (Roche) and incubated for 30 min on ice. Cellular lysate, containing the desired protein, was obtained following homogenization of the cell suspension by 20 strokes with a 1ml dounce homogenizer (Wheaton).

2.2.5 Sucrose density sedimentation analysis of VP7 soluble and particulate proteins

Soluble VP7 proteins were separated from particulate VP7 by fractionation of cellular lysate on sucrose gradients. Cellular lysate, harvested as described in section 2.2.4, was layered onto a 50-70% (w/v) discontinuous sucrose density gradient in 0.01M STE (10mM Tris, 50mM EDTA, 10mM NaCl, pH=7,4) and centrifuged at 109368 xg (30 000rpm) for 18 hours at 4°C in a Beckman SW55Ti rotor. Five hundred microlitre fractions were collected from

the bottom of the ultracentrifuge tube and the pellet resuspended in 500µl of 0.01M STE. Fractions were stored at -20°C until further use. Any variations on this protocol will be documented in the appropriate results section.

Sucrose density sedimentation analysis was also utilized to confirm the presence of AHSV VP7 trimers as described for BTV VP7 trimers (Limn & Roy, 2003, Limn et al., 2000). Cellular lysate, harvested as described in section 2.2.4, was layered onto a 10-50% (w/v) discontinuous sucrose density gradient in 0.01M STE (10mM Tris, 50mM EDTA, 10mM NaCl, pH=7,4) together with 0.5mg Bovine serum albumin (Sigma-Aldrich), 0.5mg L-lactate dehydrogenase (Roche), 1mg Catalase (Sigma) and centrifuged at 303800 xg (50 000rpm) for 18 hours at 4°C in a Beckman SW55Ti rotor. One hundred and sixty six microlitre (166µl) fractions were collected from the bottom of the ultracentrifuge tube and the pellet resuspended in 166µl of 0.01M STE. Fractions were stored at -20°C until further use.

2.2.6 VP7 protein analysis via SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein electrophoresis was performed in polyacrylamide gels under denaturing conditions, allowing separation of proteins based on their molecular sizes. Size markers Rainbow™ coloured protein molecular weight marker (Amersham Pharmacia) or the PageRuler™ prestained protein ladder (Fermentas) as well as the appropriate controls were run alongside the protein samples of interest. The 0.75 mm thick 12% separating gel, containing 0.375 M Tris-HCl pH 8.8 and 0.1% SDS, and the 5% stacking gel, containing 0.125 M Tris-HCl pH 6.8 and 0.1% SDS, were both prepared from a stock solution of 30% acrylamide, 0.8% bisacrylamide (Merck). The gels were polymerised chemically by the addition of 0.008% (v/v) N,N,N',N' tetramethylethylenediamine (TEMED) (Sigma Life Science) and 0.08% (m/v) ammonium persulphate (Roche) followed by incubation at 37°C for 20 min. In order to analyze the protein content of sucrose gradient fractions and pellet, equal quantities of the pellet and sucrose gradient fractions were resuspended in 10µl 2×Protein solvent buffer (125mM Tris-HCl pH=6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol; 0.002% bromophenol blue). These samples were denatured at 95°C for 2 minutes prior to electrophoresis. Protein samples to be analyzed for the presence of trimers were incubated at room temperature (25°C), rather than at 95°C, prior to SDS polyacrylamide electrophoresis in a modified version of the protocol first described by (Monastyrskaya et al., 1997). Electrophoresis was performed in 1 × TGS buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% SDS) using the mid-gel Hoefer electrophoresis system and applying a current of 130V for approximately 2,5 hours. The polyacrylamide gel was subsequently stained in Coomassie Brilliant Blue staining solution (0.125% Coomassie Blue, 50% methanol, 10% acetic acid) for 30 min at room temperature and destained in 5% methanol, 5% acetic acid at room temperature overnight.

2.2.7 Immunoblot analysis of VP7 protein

Immunoblot analyses were performed in order to confirm the identity and position of the wild type or modified VP7 protein on a SDS-PAGE gel. Protein samples were subjected to electrophoresis in 12% SDS polyacrylamide gels (2.2.6). One piece of Hybond C extra nitrocellulose membrane (Amersham) and two pieces

of Whatmann 3 MMChr filter paper were cut to the size of the unstained polyacrylamide gel, and together with the gel, were soaked in 1 × Towbins transfer buffer (25 mM Tris, 192 mM Glycine pH 8.3) for 30 min. Two Scotch Brite™ Sponge pads were squeezed and soaked twice in the transfer buffer. A blotting sandwich was assembled in an EC 140 Mini electroblot module, starting on the frame stand with stainless steel grid cathode. The first Scotch brite™ sponge pad was placed on the grid cathode, followed by a sheet of soaked filter paper and the polyacrylamide gel. The soaked nitrocellulose membrane was placed directly on the gel, followed by another sheet of soaked filter paper. To ensure that no air-bubbles were trapped between the layers, a pipette was rolled over the stack after each step. After the second soaked Scotch brite™ sponge pad completed the assembly, the blotting stack was covered with the palladium wire anode, turned upright and clamps attached to the sides of the module. Transfer buffer was added until the compartment was full, the assembled module was inserted into the outer tank and the safety cover placed on top. The transfer process was performed at 15 V for 45 min and 24 V for another 45 min. Satisfactory transfer was confirmed by staining the gel with Coomassie Brilliant Blue staining solution as in 2.2.11. A replica of each 12% SDS polyacrylamide gel used for Immunoblot analysis was subjected to electrophoresis under the same conditions and stained with the Coomassie Brilliant Blue staining solution as a control.

After transfer, the membrane was rinsed for 5 min in 1 × PBS. Following the addition of blocking solution (1% (m/v) milk powder in 1 × PBS), the membrane was incubated at room temperature for 1 hr to block the non-specific binding sites. The blocked membrane was sealed in a plastic bag and incubated in a primary antibody solution. Incubation was performed overnight at room temperature with gentle agitation to allow binding of the antibodies to immobilised protein. The membrane was removed and washed three times with wash buffer (0.05% Tween® 20 (Sigma Life Science) in 1 × PBS), 5 min for each wash. A secondary antibody Peroxidase-conjugated Protein A (ICN), specific for the primary antibody and labelled with the enzyme horseradish peroxidase, was diluted 1:1000 in blocking solution and added to the membrane. After incubation at room temperature for 1 hr with agitation the membrane was washed three times in wash buffer, 5 min for each wash, and once in 1 × PBS. The enzyme substrate solution was prepared as follows: A solution containing 60 mg 4-chloro-1-naphtol (Sigma Life Sciences) in 20 ml ice-cold methanol (Merck) and a solution containing 60 µl hydrogen peroxide (BDH chemicals) in 100 ml 1 × PBS were mixed together, just prior to use. The membrane was transferred to the enzyme substrate and incubated at room temperature until bands became visible. To stop the colour precipitation reaction, the membrane was rinsed with dH₂O.

2.2.8 Quantification of the relative amount of VP7 protein in the soluble fraction

The relative amount of soluble VP7 protein in the sucrose gradients was calculated from both the Coomassie blue stained PAGE gels and/or the corresponding immunoblot membranes by scanning the VP7 bands in the pellet and gradient fractions using the EZQuant software package (EZQuant Ltd). The sum of the scanned

values in the soluble fractions of the gradient, normally fractions 7-9 in a 10 fraction gradient, was then expressed as a percentage of the total VP7, which was the sum of the scanned VP7 values in the pellet and all the gradient fractions.

2.2.9 Scanning Electron Microscopy (SEM) of particulate VP7

The bottom fractions of a sucrose density gradient (Fractions 1-3) were diluted 1/5 in 1×PBS, the VP7 protein particles pelleted at 5000rpm for 45 min, resuspended in 200ul 1×PBS and left for 15 min in a fixative solution (0.15 M Na₂HPO₄, 0.1% glutaraldehyde) before being filtered through a 0.22 µm nylon filter (Schleicher & Shuell). Three subsequent washing steps with 0.075 M Na₂HPO₄ followed with a 10 min waiting step between each. The particle covered filters were then dehydrated for 15 min consecutively in 50%, 70%, 90% and three times in 100% ethanol. Critical point drying with liquid CO₂ further dehydrated the samples before being placed on an aluminium stub and sputter coated with gold-palladium in a SEM autocoating unit E5200 (Polaron Equipment Ltd.). Samples were then viewed in a Jeol 840 Scanning electron microscope.

2.2.10 Antigen preparation for the guinea pig trial

A 200ml spinner culture of Sf9 cells (2×10^8 cells) was infected with recombinant baculovirus expressing VP7 protein at an m.o.i of 5pfu/ml. Cells were harvested at 48 hours post infection by centrifugation at 829 xg (2000rpm) for 5min at 4°C. The cell pellet was resuspended in sterile lysis buffer (0.01M STE, 0.5% NP-40) and incubated for 30 min on ice. The cells were homogenised with a 7ml large dounce (Wheaton) using twenty strokes and the nuclei removed from the cellular lysate by centrifugation at 466 xg (1500rpm) for 3 min at 4°C. The cellular lysate was subsequently centrifuged through a 50-70% (w/v) discontinuous sucrose gradient in 0.01M STE at 72128 xg (20000rpm) for 18 hours at 4°C in a Beckman SW28 rotor to separate the particulate and soluble protein. The gradient was fractionated using a peristaltic pump P1 (Pharmacia Fine chemicals) and the pellet resuspended in sterile 0.01M STE buffer. The fractions and pellet were analysed for the presence of protein by 12% SDS-PAGE (2.2.6). Sucrose fractions containing the soluble VP7 protein were combined, as were the fractions containing VP7 particulate protein, and both pooled samples dialysed against sterile 0.01M STE buffer in SnakeSkin™ dialysis membrane (Pierce) with three buffer changes each for 48 hours at 4°C. The dialysed protein samples were freeze-dried in the Freezone 2.5 freeze-drier (Labconco) for 72 hours and reconstituted in sterile 1xPBS. Dilution series' of the VP7 protein samples were run on SDS-PAGE gels with known quantities of Bovine serum albumin (BSA) protein. The intensities of the BSA and VP7 protein bands were determined using the EZQuant quantification software program (EZQuant Ltd) and a standard curve drawn using the BSA protein band intensity values. The quantities of VP7 protein on the SDS-PAGE gels were extrapolated from the standard curve and the VP7 concentration calculated. Protein samples were diluted to the appropriate concentration for animal trials and frozen away at -70°C.

2.2.11 Guinea pig immunization protocol

The guinea pig trials were conducted under the strict supervision of the Onderstepoort Animal Ethics Committee according to national standards. The studies were performed at the Transboundary Animal Diseases Program (TADP) of the Onderstepoort Veterinary Institute (OVI). Separate vaccines were formulated by combining the VP7 antigen solutions with an equal volume of either complete or incomplete Freund's adjuvant. Female guinea pigs (500-600g) were inoculated subcutaneously with 40µg of soluble VP7-177(P₁)-FMDVepi protein in a final volume of 500µl which included either 250ul complete or incomplete Freund's adjuvant. Forty micrograms of particulate VP7-177(P₁)-FMDVepi or particulate VP7-177(P₁) vector protein was administered in the presence of complete Freund's adjuvant. Boost inoculations, with the same antigen dose and adjuvant mixtures, were administered three weeks after the primary inoculation. Guinea pigs were bled prior to immunization (pre-bleed), before the boost inoculation at 21 days and three weeks after the boost inoculation on day 42 of the animal trial.

2.2.12 Analysis of FMDV antibodies by ELISA and virus neutralization

FMDV ELISA and virus neutralization assays were performed at the Transboundary Animal Diseases Program (TADP) of the Onderstepoort Veterinary Institute (OVI) by Dr Jan Esterhuysen. Sera from the experimental animals were tested for the presence of anti-FMDV antibodies via indirect ELISA. SAT2/ZIM/7/83 was added to 96 well microtiter plates previously coated with rabbit anti-ZIM/7/83 antiserum. The guinea pig sera were mixed with equal volumes of negative bovine serum prior to dilution onto the plates. Horseradish peroxidase-conjugated rabbit anti-guinea pig IgG (Sigma-Aldrich), diluted 1:100 in casein, was added for 1 h at 37°C and the plates washed with PBS-Tween-80. The substrate solution, 3,3',5,5' tetramethylbenzidine (TMB) sodium phosphate/citric acid buffer and H₂O₂ was added and incubated for 15 min at room temperature. The reactions were stopped with 1 M H₂SO₄ and the optical density (OD) at 492 nm was measured with a Labsystems Multiscan Plus Photometer. Titration endpoints are expressed as the log₁₀ reciprocal of the highest dilution at which the signal obtained is twice the signal of the negative control. Under these conditions pre-bleed sera and negative controls had ELISA titers of 1.3 or less.

The presence of neutralizing FMDV antibodies in the sera of VP7-177(P₁)-FMDVepi inoculated animals was detected via cross-neutralization assays in micro-titer plates on IB-RS-2 cells as described in the OIE Manual of Standards (2009). The neutralization assays used SAT2/ZIM 7/83 to determine the presence and titers of the FMDV neutralizing antibodies. The titers were expressed as the log₁₀ reciprocal of the last dilution of serum to neutralize 100 TCID₅₀ in 50% of the wells (Rweyemamu et al., 1978). All the data was statistically analyzed by a two sample t-test assuming unequal variance.

Table 2.1: Primers used in construction of VP7 vectors, VP7 fusion proteins and VP7L345R mutants.

VP7 vectors	Primer ⁽¹⁾	5' to 3' sequence ⁽²⁾	RE Sites
VP7-144(P ₁)	O1F	GGCTCTAGAGTCGACCAAGGTCGAACGCGTGG	<i>XbaI-SalI</i>
	O2R	GGCTCTAGAAAGCTTCGGTACGTAATATCTGCC	<i>XbaI-HindIII</i>
VP7-177(P ₁)	O3F	GCTCTAGAGTCGACAGGGGGACGCAGTCATG	<i>XbaI-SalI</i>
	O4R	GCTCTAGAAAGCTTCCTTGGGGCTAGCAGCGC	<i>XbaI-HindIII</i>
VP7-200(P ₁)	O5F	GCTCTAGAGTCGACTCACTTGAGAGCGCTCC	<i>XbaI-SalI</i>
	O6R	GCTCTAGAAAGCTTCGCACCTTGAGGATCAC	<i>XbaI-HindIII</i>
VP7-144(P ₂)	O7F	GGCGAATTCCTCGAGCAAGGTCGAACGCGTGG	<i>EcoRI-XhoI</i>
	O8R	GGCGAATTCCTCGAGCAAGGTCGAACGCGTGG	<i>EcoRI-SmaI</i>
VP7 external	O9F	CACAGATCTTTCGGTATAGGATGGACGCG	<i>BglII</i>
	O10R	CACAGATCTGTAAGTGATTTCGGTATTGAC	<i>BglII</i>

VP7 fusion protein	Primer ⁽¹⁾	5' to 3' sequence ⁽²⁾	RE Sites
VP7-177(P ₁)-FMDVepi	O11F	CGGAAGCTTAGATACAACGGTGAGTGC	<i>HindIII</i>
	O12R	CGGGT CGAC GGTAGACGGGAGTTTGTG	<i>SalI</i>
VP7-144(P ₂)-FMDVepi	O13F	CGGGAATTCAGATACAACGGTGAGTGC	<i>EcoRI</i>
	O14R	CGGCT CGAG GGTAGACGGGAGTTTGTG	<i>XhoI</i>
VP7-144(P ₂)-VP2(110aa)	O15F	GGCGAATTCGATAAGCAGGGG	<i>EcoRI</i>
	O16R	GGCCT CGAG TGGTTGTCCTCC	<i>XhoI</i>
VP7-177(P ₁)-VP2epiA	O17F	GGGAAGCTTAAGAAGAAAGAAGAGGGTGAG	<i>HindIII</i>
	O18F	GGGGT CGAC CCATGCTTTTCTTATCTCCTG	<i>SalI</i>
VP7-177(P ₁)-VP2epiB	O19F	GGGAAGCTTGTAGATGTTGATCCGAATAAG	<i>HindIII</i>
	O20R	GGGGT CGAC CGCTTTCTCAATTTTTCGGT	<i>SalI</i>
VP7-177(P ₁)-VP2(150aa)	O23F	CCCAAGCTTAAAGGTCCACTGAATGACTTACGAGTTAAATTG	<i>HindIII</i>
	O24R	CCCGT CGAC TGGCATGGTTGTCCTCCATTTTCG	<i>SalI</i>
VP7-177(P ₁)-fELDKWaf	O25F	CCCAAGCTTGAATTCAGGTTACTTGAAGACTCG	<i>HindIII</i>
	O26R	CCCGT CGAC GCCTCCTACTATCATGAT	<i>SalI</i>
VP7-177(P ₁)-fRILaf	O27F	CCCAAGCTTGCTATAGAGGCGCAACAG	<i>HindIII</i>
	O28R	CCCGT CGAC GAATTCACAGCAGTGGTGCAGAT	<i>SalI</i>
VP7-177(P ₁)-RCL3R	O29F	CCCAAGCTTAGAAGAAGGAGACGAAGACTAGGGCTTTGGGGCTGC	<i>HindIII</i>
	O30R	CCCGT CGAC TCTTCTCGTCTTCGGCGGCAGATGATTTTCC	<i>SalI</i>

VP7 L345R mutants	Primer ⁽¹⁾	5' to 3' sequence ⁽²⁾	RE Sites
VP7-L345R, VP7-177(P ₁)-L351R	O21F	CAGCCCGGGCAGCCTACCACTAGTGGC	<i>SmaI</i>
	O22R	CAGCCCGGGCAAGAGCTCTGTTGATCT	<i>SmaI</i>

⁽¹⁾ Primer orientation forward (F), reverse (R), ⁽²⁾ RE sites highlighted in bold

Table 2.2: The modified VP7 proteins investigated in this study, the modifications and the authors responsible

VP7-144(P ₁)	Amino acids KLSRVD inserted downstream of amino acid 144 of AHSV-9 VP7	Dr Q. Meyer
VP7-177(P ₁)	Amino acids KLSRVD inserted downstream of amino acid 177 of AHSV-9 VP7	Dr F. Maree (Maree, 2000)
VP7-200(P ₁)	Amino acids KLSRVD inserted downstream of amino acid 200 of AHSV-9 VP7	Dr F. Maree (Maree, 2000)
VP7-144(P ₂)	Amino acids PGEFLE inserted downstream of amino acid 144 of AHSV-9 VP7	Ms J. Riley (Riley, 2003)
VP7-144(P ₂)-200(P ₁)	Amino acids PGEFLE inserted downstream of amino acid 144 of vector VP7-200(P ₁)	Ms J Riley (Riley, 2003)
VP7-144(P ₂)-177(P ₃)-200(P ₁)	Amino acids LQRPAR inserted downstream of amino acid 177 of vector VP7-144(P ₂)-200(P ₁)	Mr R. van Rensburg (van Rensburg, 2004)
VP7-L345R	Amino acid valine (V) substituted with alanine (A) in position 344 of VP7 and amino acid leucine (L) substituted with arginine (R) in position 345 of VP7	Dr Q. Meyer (Meyer, 2002)
VP7-177(P ₁)-L351R	Amino acid valine (V) substituted with alanine (A) in position 350 of VP7-177(P ₁) and amino acid leucine (L) substituted with arginine (R) in position 351 of VP7-177(P ₁)	Dr Q. Meyer (Meyer, 2002)
VP7-177(P ₁)-FMDVepi	Amino acids RYNGECKYTQQSTAIRGDRAVLAALKYANTKHKLPST (aa129-164) of the FMDV vaccine strain SAT2/ZIM/7/83 VP1 inserted into vector VP7-177(P ₁)	Dr W. Fick
VP7-144(P ₂)-FMDVepi	Amino acids RYNGECKYTQQSTAIRGDRAVLAALKYANTKHKLPST (aa129-164) of the FMDV vaccine strain SAT2/ZIM/7/83 VP1 inserted into vector VP7-144(P ₂)	Ms D. Bolton

VP7-177(P ₁)-VP2epiA	Amino acids KKKEEGEDDTARQEIRKAW (aa321-339) of virulent AHSV-4 VP2 inserted into vector VP7-177(P ₁)	Mr N. Smalle (Smalle, 2006)
VP7-177(P ₁)-VP2epiB	Amino acids VDVPNKGKWKKEHIKEVTEKLKKA (aa377-400) of virulent AHSV-4 VP2 inserted into vector VP7-177(P ₁)	Mr N. Smalle (Smalle, 2006)
VP7-144(P ₂)-VP2epiB	Amino acids DPNHDTWKNHVKEIRERMQKEQSAN (aa377-400) of AHSV-9 VP2 inserted into vector VP7-144(P ₂)	Ms J. Riley (Riley, 2003)
VP7-144(P ₂)-VP2(110aa)	Amino acids DKQGNISIEKLVEYFDLTTTFVHAKKKEEGEDDTAR QEIRKAW VKGMPYMDFSKPMKITRGFNRLFLAA LDSFRKRNGVDVPNKGKWKKEHIKEVTEKLKKA LDE (aa297-408) of virulent AHSV-4 VP2 inserted into vector VP7-144(P ₂)	Dr Q. Meyer
VP7-177(P ₁)-VP2(150aa)	Amino acids KGPLNDLRVKIERDDLSRETIIQIEYGKKFNSSAGDKQGNISIEKLVEY FDLTTTFVHAKKKEEGEDDTAR QEIRKAW VKGMPYMDFSKPMKITR GFNRNMLFLAALDSFRKRNGVDVPNKGKWKKEHIKEVTEKLKKA LDE (aa261-410) of virulent AHSV-4 VP2 inserted into vector VP7-177(P ₁)	Current study
VP7-144(P ₂)-eGFP	enhanced Green Fluorescent Protein (eGFP) (239aa) inserted into vector VP7-144(P ₂)	Mr T. Semeco
VP7-177(P ₁)-eGFP	enhanced Green Fluorescent Protein (eGFP) (239aa) inserted into vector VP7-177(P ₁)	Mr. E. Mizrachi (Mizrachi, 2008)
VP7-200(P ₁)-eGFP	enhanced Green Fluorescent Protein (eGFP) (239aa) inserted into vector VP7-200(P ₁)	Dr Q. Meyer
VP7-144(P ₂)-ELDKWA	Amino acids ELLELDKWASLW (aa652-663) of HIV-1 subtype B gp41 inserted into vector VP7-144(P ₂)	Dr Q. Meyer (Meyer, 2002)
VP7-177(P ₁)-ELDKWA	Amino acids ELLELDKWASLW (aa652-663) of HIV-1 subtype B gp41 inserted into vector VP7-177(P ₁)	Dr Q. Meyer (Meyer, 2002)
VP7-144(P ₂)-177(P ₃)- RVLA-200(P ₁)	Amino acids RVLAIERYLKD (aa560-570) of HIV-1 subtype C gp41 inserted vector VP7-144(P ₂)-177(P ₃)-200(P ₁)	Mr R. van Rensburg (van Rensburg, 2004)
VP7-144(P ₂)-177(P ₃)- (RVLA) ₄ -200(P ₁)	Amino acids RVLAIERYLKDLHRVLAIERYLKDLHRVLAIERYLKDLHRVLAIERYL KD (4 repeats of aa 560-570) of HIV-1 subtype C gp41 inserted into vector VP7-144(P ₂)-177(P ₃)-200(P ₁)	Mr R. van Rensburg (van Rensburg, 2004)

VP7-177(P ₁)- f ₁ ALDSWKf ₂	$\longleftrightarrow$ f₁ $\longleftrightarrow$ f₂ Amino acids RLLED SQNQKEKNEKDLLALDSWKNLWNWFDITNWLWYIKIFIMIVGG (aa625-672) of HIV-1 subtype C gp41 inserted into vector VP7-177(P₁)	Ms D. Rutkowska (Rutkowska, 2002)
VP7-177(P ₁)- f ₃ ELDKWf ₄	$\longleftrightarrow$ f₃ $\longleftrightarrow$ f₄ Amino acids TLIEESQNQQEKNEQELLEELDKWANLWNWFDITNWLWYIKIFIMIIGG (aa637-684) of HIV-1 subtype B gp41 inserted into vector VP7-177(P₁)	Current study
VP7-177(P ₁)- f ₅ RVLAf ₆	$\longleftrightarrow$ f₅ $\longleftrightarrow$ f₆ AIEAQQHMLQLTVWGVKQLQTRVLAIERYLKDQQLGLWGCSGKIICTTAV (aa539-589) of HIV-1 subtype C gp41 inserted into vector VP7-177(P₁)	Ms D. Rutkowska (Rutkowska, 2002)
VP7-177(P ₁)- f ₇ RILAf ₈	$\longleftrightarrow$ f₇ $\longleftrightarrow$ f₈ AIEAQQHLLRLTVWGIKQLQARVLAVERYLKDQQLGLWGCSGKLICTTTV (aa551-601) of HIV-1 subtype B gp41 inserted into vector VP7-177(P₁)	Current study
VP7-177(P ₁)- f ₅ RVLAf ₆ f ₁ AL DSWKf ₂	$\longleftrightarrow$ f₅ $\longleftrightarrow$ f₆ $\longleftrightarrow$ f₁ AIEAQQHMLQLTVWGVKQLQTRVLAIERYLKDQQLGLWGCSGKIICTTAVEFRLLD $\longleftrightarrow$ f₁ $\longleftrightarrow$ f₂ SQNQKEKNEKDLLALDSWKNLWNWFDITNWLWYIKIFIMIVGG (aa539-589 and aa625-672) of HIV-1 subtype C gp41 inserted into vector VP7-177(P₁)	Ms D. Rutkowska (Rutkowska, 2002)
VP7-200(P ₁)- f ₅ RVLAf ₆ f ₁ AL DSWKf ₂	$\longleftrightarrow$ f₅ $\longleftrightarrow$ f₆ $\longleftrightarrow$ f₁ AIEAQQHMLQLTVWGVKQLQTRVLAIERYLKDQQLGLWGCSGKIICTTAVEFRLLD $\longleftrightarrow$ f₁ $\longleftrightarrow$ f₂ SQNQKEKNEKDLLALDSWKNLWNWFDITNWLWYIKIFIMIVGG (aa539-589 and aa625-672) of HIV-1 subtype C gp41 inserted into vector VP7-200(P₁)	Ms H. Kretzmann (Kretzmann, 2006)
VP7-177(P ₁)- RCL3R	Amino acids RRRRRRLGLWGCSGKIICRRRRRR aa574-585 of HIV-1 subtype C gp41 inserted into vector VP7-177(P₁) with 6 arginine residues flanking the N and C terminals	Current study

2.3 Results

2.3.1 Solubility of the wild type AHSV-9 VP7 protein

At the start of the investigation it was important to determine what percentage of the wild type (WT) AHSV VP7 protein, expressed in Sf9 insect cells by recombinant baculovirus, was present in a soluble form. To investigate Sf9 cells were infected with a recombinant baculovirus expressing the AHSV WT VP7 protein, harvested at 72 hours post infection (p.i.), as described in the Materials and methods section (2.2.4), and the soluble and particulate protein fractions within the cellular lysate separated via centrifugation through sucrose density gradients (2.2.5). The sucrose gradient fractions were analyzed for the presence of VP7 protein by SDS-PAGE (2.2.6) and immunoblot analysis with a VP7-specific primary antibody (2.2.7). The result is shown in Fig 2.2.

A protein band, approximately 37.9 KDa in size, was visible on the SDS-PAGE gel within fractions 1-7, as well as in the pellet fraction, of the sucrose density gradient. This was the expected size of the wild type VP7 protein. In order to confirm the VP7 identity of the 37.9 KDa protein band, an immunoblot was performed on a replica SDS-PAGE gel with a VP7-specific primary antibody. A VP7-specific protein band, approximately 37.9KDa in size, was detected in fractions 1-10 and the pellet of the sucrose gradient. Under the experimental conditions specified, the bulk of the AHSV WT VP7 protein was insoluble and sedimented to the pellet of the sucrose gradient during centrifugation. The VP7 proteins, that co-sedimented with the soluble cellular proteins in fractions 8-10, were considered the soluble fraction. The soluble WT VP7 protein could, however, only be detected following the immunoblot procedure and represents only a small fraction of the total amount of VP7 protein expressed in insect cells at 72 hours post-infection. The relative amount of soluble VP7 protein in fractions 8-10 was calculated using the EZQuant software package (EZQuant Ltd), as described in the Materials and Methods section (2.2.8), and was less than 9% of the total WT-VP7 protein expressed. Next we investigated whether the solubility of the AHSV VP7 protein was affected by the insertion of 6-mer peptides into this proteins top domain.

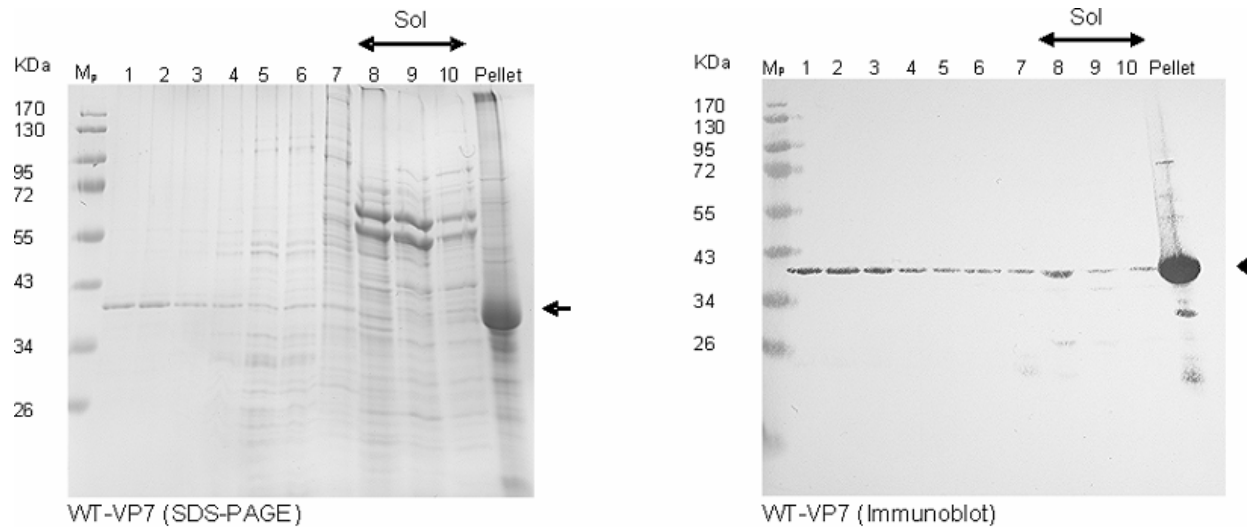


Fig 2.2: SDS-PAGE and immunoblot of sucrose gradient fractions following sedimentation analysis of cellular lysates containing the wild type (WT) AHSV-9 VP7 protein (37.9 KDa). Cellular lysate was layered onto a 50-70% sucrose gradient and centrifuged in a Beckman SW 55Ti rotor for 18h at 109368 xg (30 000rpm). Fractions were collected from the bottom of the tube and equal quantities of each fraction and pellet analyzed by SDS-PAGE and Coomassie Blue staining as well as immunoblotting using guinea pig anti-VP7 antibodies. Double-sided arrows at the top of the gel/membrane indicate the soluble fractions of the gradient. The arrow on the side indicates, in each case, the position of the VP7 protein on the gel/membrane. Marker protein (M_p) was the PageRuler™ Prestained protein ladder (Fementas). The relevant sizes are indicated in KDa. Lanes 1-10 contain sucrose fractions 1-10 of the sucrose density gradient, respectively.

2.3.2 Solubility of top domain modified AHSV VP7 vector proteins

The level of protein expression within the cell has an effect on VP7 protein solubility and is influenced by a range of factors including the multiplicity of infection (m.o.i) and the time post infection (p.i) at which the protein is harvested (Mizrachi, 2008). The solubility of the VP7 vector proteins was, therefore, compared under the same experimental conditions in this study in order to detect any differences in protein solubility. Vector proteins VP7-144(P₂), VP7-144(P₁), VP7-177(P₁), VP7-200(P₁), VP7-144(P₂)-200(P₁) and VP7-144(P₂)-177(P₃)-200(P₁) (Table 2.2) were harvested at 72 hours p.i from recombinant baculovirus-infected Sf9 cells and their solubility assessed via sucrose density gradient sedimentation analysis, as described in the Materials and Methods section (2.2.4-2.2.7). The relative amount of VP7 protein was determined from both the Coomassie-blue stained gels and immunoblots as described in 2.2.8.

In each case the VP7 identity of the 38.6KDa protein band observed on the SDS polyacrylamide gel was confirmed via immunoblotting with a VP7-specific antiserum (Fig 2.3). The relative amount of soluble VP7 protein was calculated from the both the Coomassie-blue stained polyacrylamide gel and the immunoblots and found to

be very similar. These solubility profiles were found to be reproducible in three, or more, independent repeats of the same experiment. Thus the immunoblot result was found to be a reliable indicator of the amount of soluble VP7 protein relative to the amount of particulate VP7 protein present.

The largest influence of peptide insertion on VP7 protein solubility was in the case of the VP7-144(P₂) vector protein (Fig 2.3; Table 2.3). The bulk of the 38.6KDa VP7-144(P₂) protein (60%) co-sedimented with the soluble cellular and viral proteins in fractions 8-10 of sucrose density gradient. Very little of this protein was observed in the pellet fraction. Thus the insertion of the P₂ peptide downstream of amino acid 144 in the WT-VP7 top domain converted the insoluble WT-VP7 to a largely soluble protein. The insertion of P₁ into the same site, however, resulted in the majority (94%) of the 38.6KDa VP7-144(P₁) vector protein being insoluble and distributed between sucrose fractions 1-7 and the pellet of its respective sucrose gradient. Similarly the insertion of P₁ downstream of amino acid 200 resulted less than 11% of the VP7-200(P₁) vector protein being soluble and located in sucrose fractions 8-11. The insertion of P₁ downstream of amino acid 177, however, yielded a more soluble protein, with the VP7-177(P₁) protein having a solubility of 28%. The insertion of additional 6-mer peptides into the VP7 top domain had a significant influence on VP7 vector protein solubility. The insertion of the P₂ peptide into the mostly insoluble VP7-200(P₁) vector increased protein solubility considerably, with approximately 46% of the VP7-144(P₂)-200(P₁) protein sedimenting in the soluble fractions 8-10. The insertion of the P₃ peptide downstream of position 177 in the mostly soluble VP7-144(P₂)-200(P₁) vector also had a significant effect with the resulting VP7-144(P₂)-177(P₃)-200(P₁) protein having virtually no soluble component. Although the amount of VP7-144(P₂)-177(P₃)-200(P₁) protein was low, possibly indicating a decrease in baculovirus stock titre, the detection of VP7-144(P₂)-177(P₃)-200(P₁) protein in sucrose fractions 1-3 and pellet of the gradient on the immunoblot membrane indicated that this protein preferentially assembled into insoluble particles and did not have a detectable soluble component.

These results gave an indication of the influence of both the inserted peptide and the peptide insertion site in determining the solubility of the VP7 protein. The insertion of different peptides into the same position within the VP7 top domain resulted in vectors VP7-144(P₁) and VP7-144(P₂) having vastly different solubility's indicating the influence of the inserted peptide. When the same peptide was inserted into different positions within the VP7 top domain, the VP7-177(P₁) and VP7-200(P₁) vectors also had differing solubility's indicating the influence of the insertion site.

It has been shown that the insertion of 6-mer peptides into the AHSV VP7 top domain influenced this proteins solubility. The effect of these insertions on the ability of the top domain modified VP7 vector proteins to form trimers was also not known and was subsequently investigated.

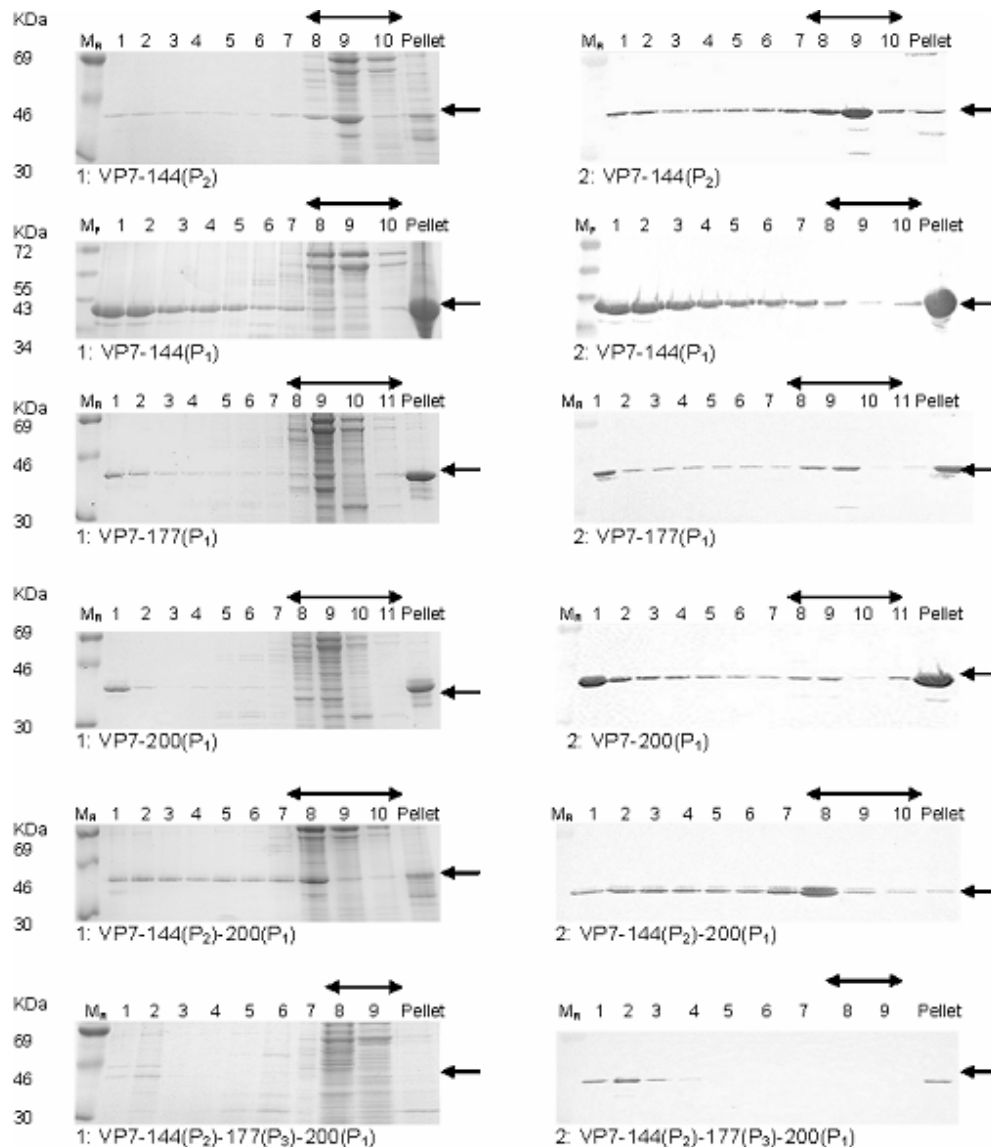


Fig 2.3: SDS-PAGE and immunoblots of the sucrose gradient fractions following sedimentation analysis of cellular lysates containing modified VP7 proteins VP7-144(P₂) (38.6 KDa), VP7-144(P₁) (38.6 KDa), VP7-177(P₁) (38.6 KDa), VP7-200(P₁) (38.6 KDa), VP7-144(P₂)-200(P₁) (39.2 KDa) and VP7-144(P₂)-177(P₃)-200(P₁) (40 KDa). Cellular lysate was layered onto a 50-70% sucrose gradient and centrifuged in a Beckman SW 55Ti rotor for 18h at 30 000rpm. Fractions were collected from the bottom of the tube and equal quantities of each fraction and pellet analyzed by SDS-PAGE and Coomassie Blue staining (1) as well as immunoblotting using guinea pig anti-VP7 antibodies (2). Double-sided arrows at the top of the gel/membrane indicate the soluble fractions of the gradient. The arrow on the side indicates, in each case, the position of the VP7 protein on the gel/membrane. Marker protein was the Rainbow™ coloured protein molecular weight marker (Amersham Pharmacia) (M_R) or the PageRuler™ Prestained protein ladder (Fermentas) (M_P). The relevant sizes are indicated in KDa. Lanes 1-11 contain sucrose fractions 1-11 of the sucrose density gradient, respectively.

Table 2.3: Relative amount of soluble VP7 protein in the cellular lysate 72 hours post infection

VP7 vectors	Percentage soluble protein at 72 hours post-infection*
Wild type VP7	9% $\pm$ 0.5%
VP7-144(P ₂)	60% $\pm$ 3%
VP7-144(P ₁)	6% $\pm$ 0.7%
VP7-177(P ₁)	28% $\pm$ 1.5%
VP7-200(P ₁)	11% $\pm$ 0.5%
VP7-144(P ₂)-200(P ₁)	46% $\pm$ 2%
VP7-144(P ₂)-177(P ₃)-200(P ₁)	0% $\pm$ 0.5%

*Calculated using the EZQuant software package (EZQuant Ltd)

2.3.3 Trimer formation of WT VP7 and top domain modified VP7 proteins

As AHSV VP7 trimer molecules were envisioned as antigen presentation system, it was important to determine whether peptide insertions into the VP7 top domain affected trimer formation. Two strategies were used to investigate the trimer status of the AHSV VP7 vector proteins. The first was a modified gradient sedimentation strategy first described by (Limn & Roy, 2003, Limn et al., 2000). This strategy was initially used to determine whether soluble BTV protein is composed of trimers. The centrifugation conditions selected ensured size-based separation of the soluble VP7 trimers and the soluble VP7 monomers. Specific size marker proteins were included within the gradient to confirm the size of the suspected VP7 trimer and monomer molecules.

The sedimentation analysis strategy is, however, only applicable to soluble VP7 proteins. In order to determine whether aggregated or insoluble AHSV VP7 protein is also composed of trimers, a modified SDS-PAGE initially described by (Monastyrskaya et al., 1997) was used in this study. These authors found that the WT AHSV VP7 trimer was stable in the presence of SDS up to a temperature of at least 37°C. As the 120KDa AHSV VP7

trimers migrated through the SDS polyacrylamide gel at a slower rate than the 38.6KDa VP7 monomer, it was possible to distinguish between the two forms of VP7.

2.3.3.1 Trimer status of soluble AHSV VP7 protein

VP7-144(P₂), as the AHSV VP7 vector protein with the largest soluble fraction, was initially selected to determine whether its soluble fraction had the sedimentation profile expected of a trimer molecule. The soluble BTV VP7 protein had previously been found to have the sedimentation profile expected of a trimer (Limn & Roy, 2003, Limn et al., 2000) and was included in this study as a positive control. The 250KDa, 140 KDa and 66 KDa protein size markers were also included in order to confirm the size of the suspected VP7 trimer and monomer molecules. The AHSV VP7 trimer and the BTV VP7 trimer are both approximately 120KDa in size and were expected to sediment in the same position within the low density sucrose gradient following sucrose density sedimentation analysis (2.2.5). The sedimentation profiles of the soluble BTV WT VP7 trimer and soluble AHSV VP7-144(P₂) protein were subsequently compared (Fig 2.4).

The sedimentation profile of the soluble VP7-144(P₂) protein was comparable to that of the soluble BTV WT VP7 trimers (Fig 2.4, fractions 8-11). This indicated that the soluble VP7-144(P₂) protein also had the sedimentation profile expected of a trimer molecule. In order to confirm the size of the VP7-144(P₂) multimer, soluble VP7-144(P₂) protein was centrifuged in the same sucrose gradient with the size marker proteins Catalase (250KDa), L-lactate dehydrogenase (140KDa) and Bovine serum albumin (66KDa). The size of the VP7-144(P₂) multimer was found to be just smaller than the 140KDa L-lactate dehydrogenase tetramer but larger than the 66KDa Bovine serum albumin protein and, thus, corresponded to the predicted size of a VP7 trimer (120KDa). It was thus concluded that the soluble VP7-144(P₂) protein was composed of trimer molecules. The insertion of the P₂ peptide downstream of amino acid 144 was therefore found not to interfere with the ability of the resulting VP7-144(P₂) protein to form trimers. The sedimentation profiles of soluble VP7-177(P₁), VP7-200(P₁) and VP7-144(P₂)-200(P₁) vector proteins, following a similar sucrose gradient sedimentation strategy, were subsequently compared to that of the established soluble VP7-144(P₂) trimers (Fig 2.5).

The soluble VP7-177(P₁), VP7-200(P₁) and VP7-144(P₂)-200(P₁) vector proteins had sedimentation profiles comparable to that of the soluble VP7-144(P₂) trimer. As the bulk of the soluble VP7-177(P₁), VP7-200(P₁) and VP7-144(P₂)-200(P₁) proteins sedimented in the same sucrose fractions as the soluble VP7-144(P₂) trimers (Fig 2.5, lanes 6-9) it was concluded that the soluble VP7-177(P₁), VP7-200(P₁) and VP7-144(P₂)-200(P₁) proteins were most likely also in the form of trimers. Thus the insertion of peptide P₁ downstream of amino acid 177 and 200, and the simultaneous insertion of peptides P₂ and P₁ downstream of amino acids 144 and 200, respectively, did not interfere with the ability of the resulting VP7 vector proteins to form trimers. The sucrose gradient sedimentation strategy could, however, only be used to assess the trimer status of soluble VP7 protein.

A modified SDS-PAGE strategy (Monastyrskaya et al., 1997) was used to assess the trimer status of aggregated or insoluble VP7 proteins, including AHSV WT-VP7, VP7-144(P₁) and VP7-144(P₂)-177(P₃)-200(P₁).

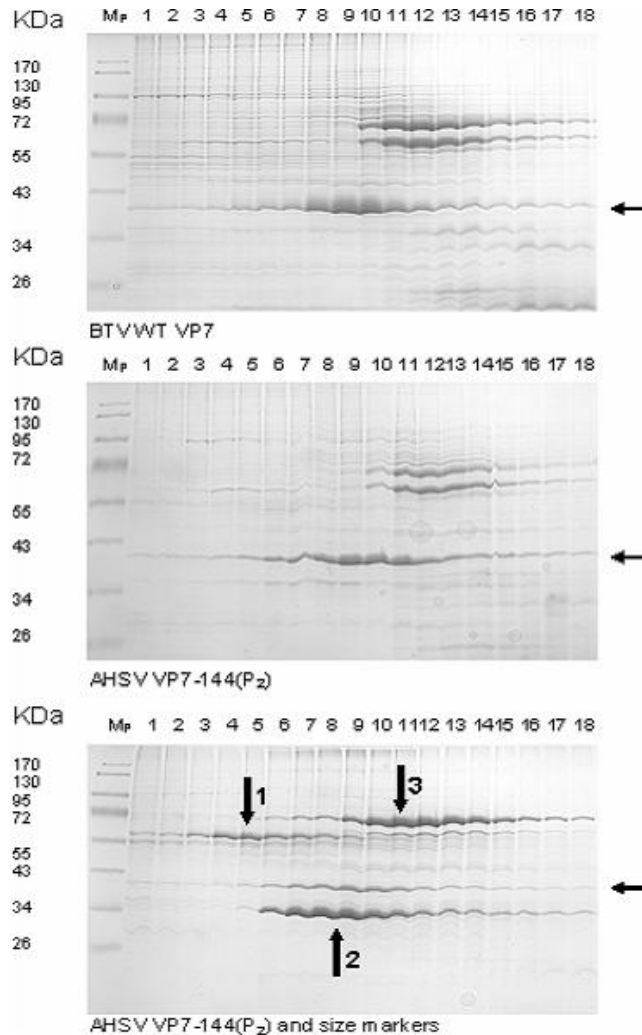


Fig 2.4: Sucrose sedimentation analysis of the soluble fractions of BTV WT-VP7 and AHSV VP7-144(P₂). Sf9 cell lysates, containing expressed VP7-144(P₂) and BTV WT-VP7 proteins, were centrifuged through 10-50% sucrose gradients in a SW 55Ti rotor at 303800 xg (50 000rpm) for 18h at 4°C. Twenty-eight fractions were collected from the bottom of the tube and equal quantities of fractions 1-18 analyzed by SDS-PAGE and Coomassie Blue staining, as shown in the first two panels. The size of the VP7-144(P₂) multimer was assessed by co-sedimentation with three size marker proteins within the same gradient. The position of the marker proteins, (1) Catalase (250 kDa), consisting of four 60-65 kDa subunits, (2) L-lactate dehydrogenase (140 kDa), consisting of four 36.5 kDa subunits and (3) Bovine serum albumin (66 kDa), are indicated with arrows. M_p is a PageRuler™ Prestained molecular size protein ladder (Fermentas). The relevant sizes are indicated in kDa. Lanes 1-18 contain sucrose fractions 1-18 of the sucrose density gradient, respectively. The arrows on the side of gel indicate the position of the VP7 protein.

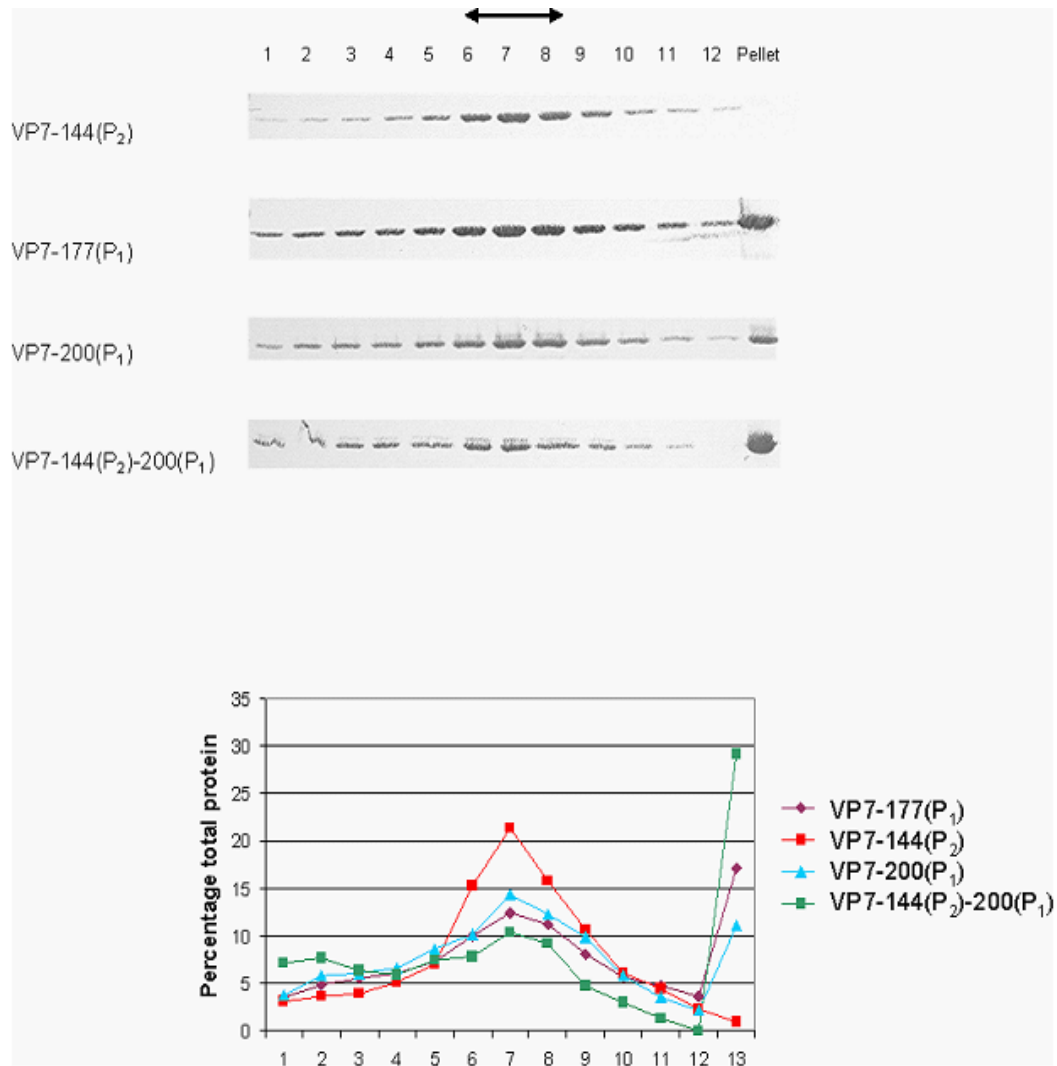


Fig 2.5: Sucrose sedimentation analysis of the soluble fractions of vector proteins VP7-144(P₂), VP7-177(P₁), VP7-200(P₁) and VP7-144(P₂)-200(P₁). Sf9 cell lysates, containing these expressed vector proteins, were centrifuged through 10-40% sucrose gradients in a SW 55Ti rotor at 50 000rpm for 21h at 4°C. Twenty-eight fractions were collected from the bottom of the tube and equal quantities of the first 12 fractions were analyzed by SDS-PAGE and immunoblotting with anti-VP7 antibodies. Lanes 1-12 contain sucrose fractions 1-12 of the sucrose density gradient, respectively. The corresponding VP7 vector protein sedimentation profiles are shown in the graph below. The double-sided arrow indicates the sucrose fractions containing the majority of the soluble VP7 protein.

2.3.3.2 Trimer status of aggregated or particulate AHSV VP7 protein

Although it has been shown that the soluble components of the VP7-144(P₂), VP7-177(P₁), VP7-200(P₁) and VP7-144(P₂)-200(P₁) vector proteins are composed of trimers, it was not known whether the aggregated or particulate components of these proteins, as well as those of the insoluble VP7-144(P₁) and VP7-144(P₂)-177(P₃)-200(P₁) proteins, were also composed of trimers. The insoluble WT VP7 protein aggregates had previously been shown to be composed of trimer molecules via a modified SDS-PAGE strategy (Monastyrskaya et al., 1997). This protocol is based the observation that AHSV WT VP7 trimers migrate as multimers on SDS polyacrylamide gels when the protein samples are heated to 37°C instead of 100°C prior to electrophoresis. A modified version of protocol (2.2.6) was used here to confirm the presence of WT VP7 trimers in the insoluble, particulate fraction of WT VP7 as well as to determine the presence of trimer molecules in the particulate fractions of the VP7-144(P₁), VP7-144(P₂), VP7-177(P₁), VP7-200(P₁), VP7-144(P₂)-200(P₁) and VP7-144(P₂)-177(P₃)-200(P₁) vector proteins (Fig 2.6).

As the soluble components of the VP7-177(P₁) and VP7-144(P₂) vector proteins were earlier shown to be composed of trimers (2.3.3.1), the high molecular weight VP7-specific protein band migrating just above the 170KDa protein size marker in soluble fractions 8-11 of their respective sucrose gradients was deemed to be soluble trimer. Although the molecular size of this protein was greater than that expected for an AHSV VP7 trimer molecule (117KDa), this size discrepancy may only be due to the trimer molecule migrating through the polyacrylamide gel in its polymeric form and is not indicative of its true molecular size. Interestingly, this high molecular weight trimer band was also observed in the sucrose fractions containing the particulate or aggregated components of the VP7-177(P₁) and VP7-144(P₂) proteins indicating that the insoluble aggregates of these proteins were also composed of VP7 trimer molecules. The presence of the high molecular weight VP7 trimer protein band in the particulate fractions of the AHSV WT VP7 sucrose gradient confirmed earlier results indicating that WT VP7 aggregates were composed of trimer molecules (Monastyrskaya et al., 1997). The presence of the high molecular weight VP7 trimer band in the sucrose fractions containing particulate VP7-144(P₁) protein indicated that this insoluble vector protein was also capable of forming trimers. In addition to the high molecular weight VP7 trimer band, a VP7-specific protein band of approximately the expected size of the VP7 monomer (39KDa), was also observed in the soluble and particulate fractions of the VP7-144(P₁) and VP7-144(P₂) vector proteins. This indicated that the trimers formed by these proteins are not as stable as those formed by the WT-VP7 and VP7-177(P₁) trimers in the presence of SDS at room temperature. In addition, the VP7-200(P₁) and VP7-144(P₂)-200(P₁) proteins, whose soluble fractions were earlier shown to be in the form of trimers (2.3.3.1), were solely in the form of monomers following electrophoresis. This indicated that these trimers were completely unstable in the presence of SDS at room temperature. It was not possible to determine whether VP7-144(P₂)-177(P₃)-200(P₁) protein formed trimers from the SDS-PAGE data.

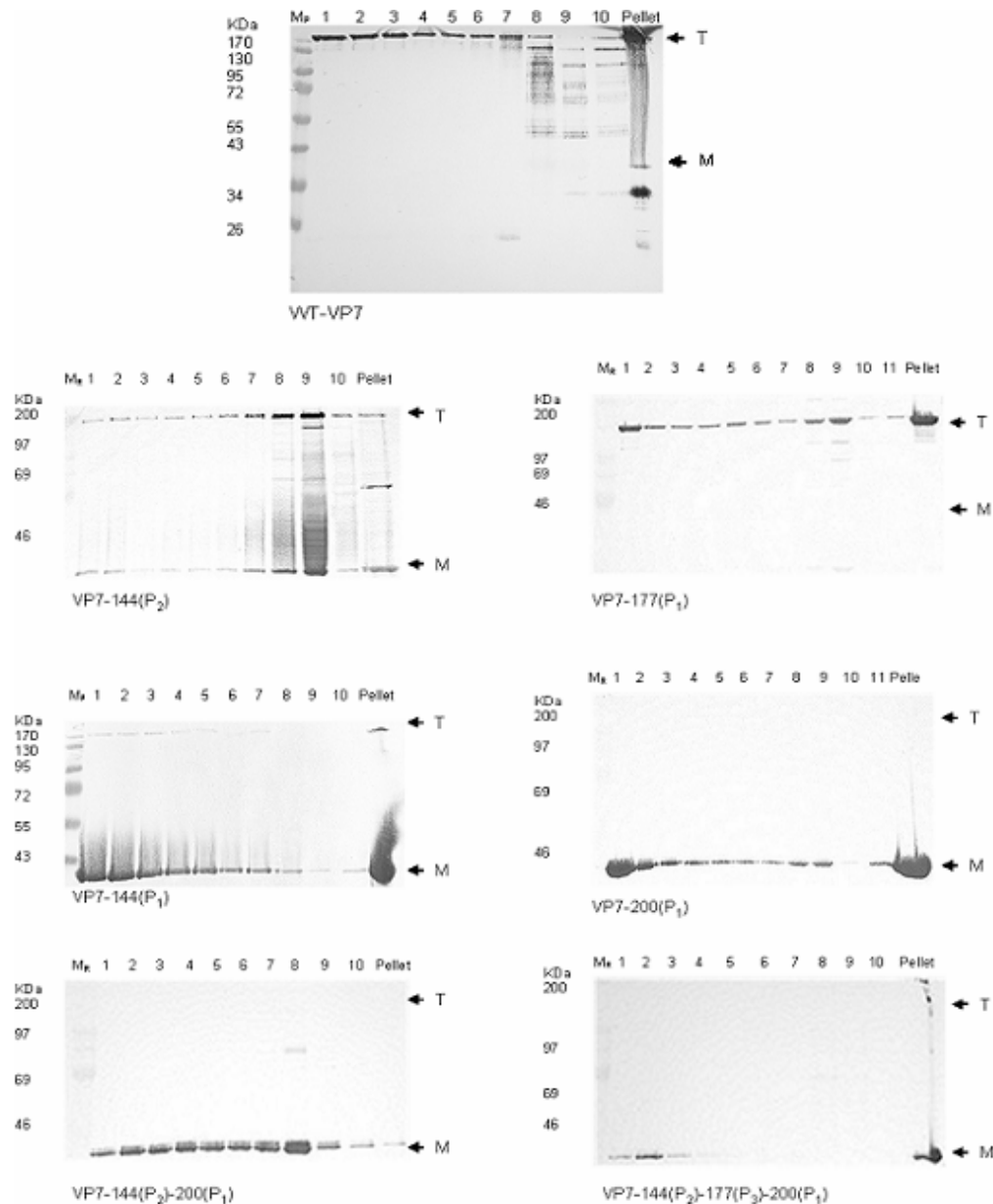


Fig 2.6: Immunoblot analyses to assess the stability of different AHSV VP7 trimers in the presence of SDS at room temperature. Sucrose gradient fractions of AHSV WT-VP7, VP7-144(P₁), VP7-144(P₂), VP7-177(P₁), VP7-200(P₁), VP7-144(P₂)-200(P₁) and VP7-144(P₂)-177(P₃)-200(P₁) were analyzed as in Fig 2.2 and 2.3, except the protein samples were incubated at room temperature rather than 95°C prior to SDS-PAGE. The VP7 proteins in the immune blot were identified with guinea pig anti-VP7 antibodies. The large VP7 multimer (and presumed trimer position) is indicated by a T and the monomer position by an M. Marker protein was the Rainbow™ coloured protein molecular weight marker (Amersham Pharmacia) (M_R) or the PageRuler™ Prestained protein ladder (Fermentas) (M_P). The relevant sizes are indicated in kDa. Lanes 1-11 contain sucrose fractions 1-11 of the sucrose density gradients, respectively.

These results indicated that although the insertion of 6-mer peptides into specific sites within the VP7 top domain did not interfere with the ability of the VP7 vector proteins to form trimers, the VP7 vectors demonstrated differences in trimer stability in SDS at room temperature.

Preliminary studies indicated that a single amino acid mutation near to the C-terminal and within the bottom domain of the AHSV VP7 protein may also affect VP7 solubility (Meyer, 2002). It was subsequently investigated whether this mutation within the C-terminals of the AHSV WT-VP7 and VP7-177(P₁) proteins influences protein solubility, trimer formation and trimer stability.

2.3.4 Solubility and trimer formation of a bottom domain modified AHSV VP7 protein

It was suggested that the substitution of a non-polar, uncharged leucine residue in position 345 of AHSV VP7 bottom domain with a polar, positively charged arginine residue, the BTV VP7 equivalent, may increase AHSV VP7 solubility (Monastyrskaya et al., 1997). Preliminary evidence indicated that both the AHSV WT-VP7 protein and the VP7-177(P₁) vector protein with this mutation were more soluble than their respective parent proteins (Meyer, 2002). Here the solubility of the mutant VP7-L345R and VP7-177(P₁)-L351R proteins was re-assessed under standardized sucrose density gradient sedimentation and quantified (2.2.5-2.2.8). The ability of VP7-L345R and VP7-177(P₁)-L351R proteins to form trimers, and the stability of any such trimers, was also assessed using the modified SDS-PAGE strategy previously described (2.3.3.2).

The L345R substitution in the bottom domain of the AHSV WT VP7 protein resulted in a significant increase in the solubility of this protein from 9% to 48% (Fig 2.7). The same amino acid substitution, in position 351 of VP7-177(P₁), increased the solubility of the vector protein from 28% to 40%. In addition to the leucine to arginine substitution, the cloning strategy also resulted in a valine to alanine substitution in positions 344 and 350 of the WT-VP7 and VP7-177(P₁) proteins, respectively. These non-polar substitutions were unlikely to have had any impact on the solubility of the mutant proteins. The presence of a high molecular weight protein bands migrating just below the 200KDa protein size marker confirmed not only that the mutant VP7-L345R and VP7-177(P₁)-L351R proteins formed trimers but that, like the WT VP7 and VP7-177(P₁) trimers, these trimers were stable in the presence of SDS at room temperature. Thus this mutation increased the solubility of the mutant VP7 protein without affecting its trimer formation and stability.

As the AHSV VP7 protein is to be used as means of delivering and presenting of foreign peptides to the immune system, it was necessary to investigate how the insertion of an antigenic peptide into the VP7 top domain would affect VP7 protein solubility, trimer formation and trimer stability.

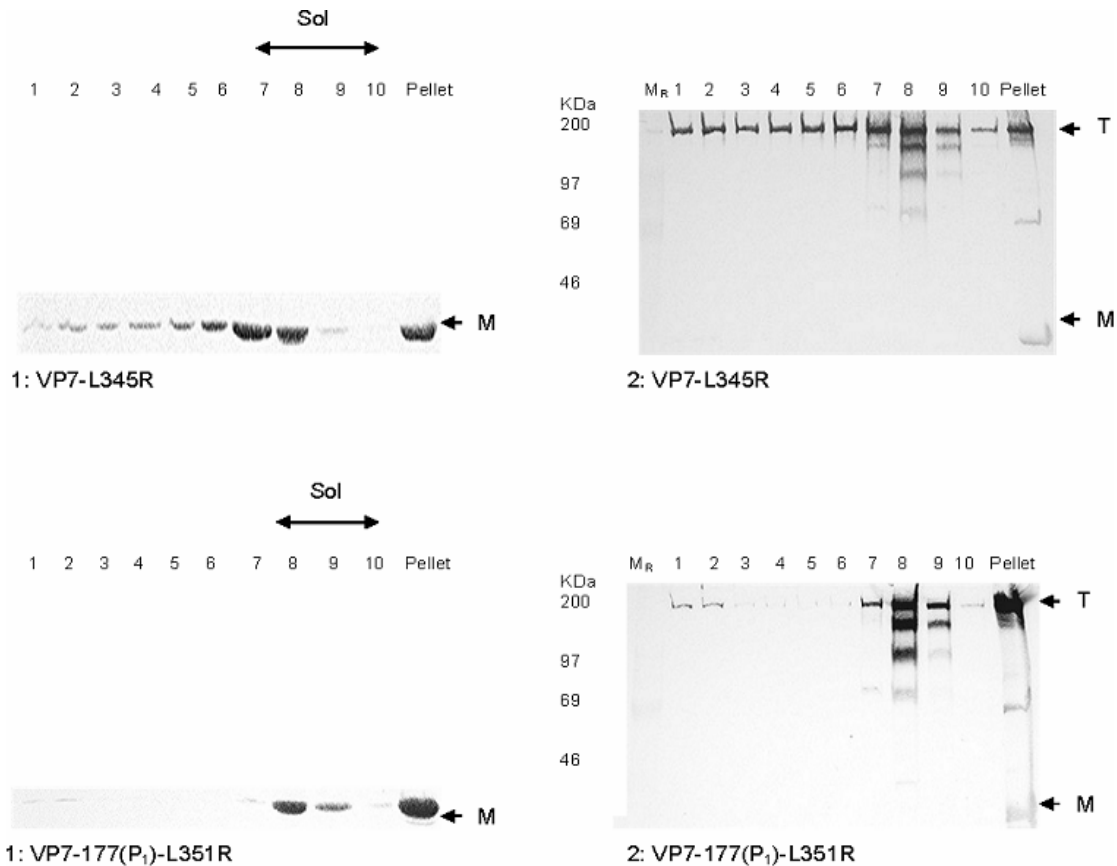


Fig 2.7: Immune blot analyses to assess the solubility and trimer stability of VP7-L345R (37.9 KDa) and VP7-177(P₁)-L351R (38.6 KDa) mutant proteins. Sucrose gradient fractions, following sedimentation analysis of the cellular lysates containing these proteins, were either incubated at 95°C prior to electrophoresis to determine protein solubility (1) or incubated at room temperature to determine trimer stability in the presence of SDS (2). Cellular lysate was layered onto a 50-70% sucrose gradient and centrifuged in a Beckman SW 55Ti rotor for 18h at 30 000rpm. Fractions were collected from the bottom of the tube and equal quantities of each fraction and pellet analyzed by SDS-PAGE. Lanes 1-10 contain sucrose fractions 1-10 of the sucrose density gradient, respectively. The VP7 proteins in the immune blots were identified with guinea pig anti-VP7 antibodies. The large VP7 multimer (and presumed trimer position) is indicated by a T and the monomer position by a M. Double-sided arrows at the top of the membrane indicate the soluble fractions of the gradient. Marker protein was the Rainbow™ coloured protein molecular weight marker (Amersham Pharmacia) (M_R). The relevant sizes are indicated in KDa.

2.3.5 Solubility and trimer formation of a VP7-FMDV fusion protein

The VP7 vector proteins have been shown to differ with regards to their solubility as well as their trimer stability. It was not known how the insertion of a larger foreign peptide into these vectors would affect these properties. Although this question is addressed mostly in chapter 3, here the effect of inserting a Foot-and-mouth disease virus VP1 peptide (aa129-164), containing the immunodominant β G- β H loop, into vector VP7-177(P₁) on VP7

solubility and trimer formation was investigated. Fusion protein VP7-177(P₁)-FMDVepi was harvested 72 hours p.i from recombinant baculovirus infected insect cells and its solubility analysed as described in the Material and Methods sections 2.2.4-2.2.8. The ability of this protein to form trimers was assessed via the modified SDS-PAGE protocol as previously described (2.3.3.2). The identity and position of the VP7 protein was confirmed by immunoblots (results not shown).

The insertion of the FMDV peptide into site 177(P₁) increased the solubility of the VP7-177(P₁) vector protein from 28% (2.3.2) to 40% (Fig 2.8). The soluble component of the VP7-177(P₁)-FMDVepi protein co-sedimented with the soluble cellular and viral proteins in sucrose fractions 6-8. Trimer analysis showed no indication of the 42KDa monomer but revealed a shift of the VP7-177(P₁)-FMDVepi monomer to a high molecular weight protein band that migrated just below the 200KDa marker. This multimer corresponded in size to the VP7-177(P₁) trimers, that have previously been observed following a modified SDS-PAGE protocol (2.3.3.2), indicating that the multimer was indeed a trimer molecule. The presence of these trimers in the soluble and particulate sucrose fractions indicated that both the soluble and particulate components of the VP7-177(P₁)-FMDVepi protein were composed of trimers and that, like VP7-177(P₁) trimers, they were stable in the presence of SDS at room temperature.

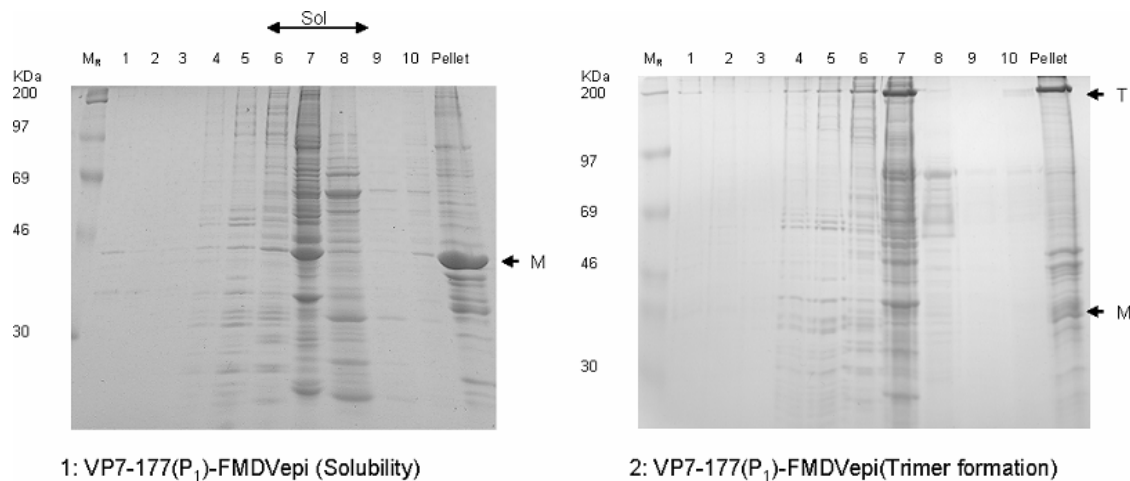


Fig 2.8: SDS-PAGE analysis to assess the solubility and trimer formation of the VP7-177(P₁)-FMDVepi protein (42KDa). Sucrose gradient fractions, following sedimentation analysis of the cellular lysate containing the protein, were either incubated at 95°C prior to electrophoresis to determine protein solubility (1) or incubated at room temperature to determine trimer formation (2). Cellular lysate was layered onto a 50-70% sucrose gradient and centrifuged in a Beckman SW 55Ti rotor for 18h at 30 000rpm. Fractions were collected from the bottom of the tube and equal quantities of each fraction and pellet analyzed by SDS-PAGE. Lanes 1-10 contain sucrose fractions 1-10 of the sucrose density gradient, respectively. The large VP7 multimer (and presumed trimer position) is indicated by a T and the monomer position by a M. The double-sided arrow at the top of the gel indicate the soluble fractions of the gradient. Marker protein was the Rainbow™ coloured protein molecular weight marker (Amersham Pharmacia) (M_R). The relevant sizes are indicated in KDa.

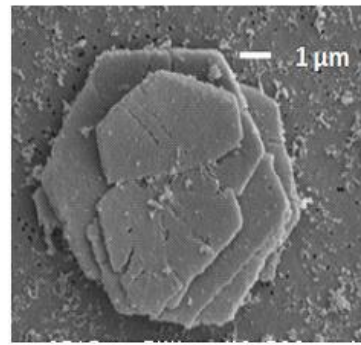
Next we investigated whether the 6-mer peptide insertions, as well as the larger 36-mer FMDV peptide insertion, had any effect on the characteristic morphology of the AHSV VP7 crystal-like particles, previously described (Burroughs et al., 1994).

2.3.6 Morphological characterization of the particulate WT VP7 and top domain modified VP7 proteins

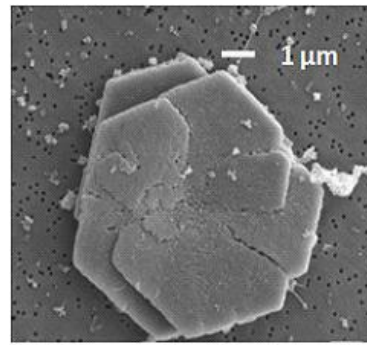
To determine whether the peptide insertions within the top domain of WT-VP7 affected the morphology of the WT-VP7 hexagonal crystal-like particles described by Chuma et al., 1992, WT-VP7, VP7-177(P₁), VP7-200(P₁), VP7-144(P₂), VP7-144(P₂)-177(P₃)-200(P₁) and VP7-177(P₁)-FMDVepi particles were analysed by scanning electron microscopy (SEM) as described in 2.2.9. The resulting images are shown in Fig 2.9.

The WT-VP7 protein was in the form of large, flat hexagonal crystal-like particles, as previously described (Burroughs et al., 1994, Chuma et al., 1992). These crystal-like particles were approximately 6-7µm in diameter and arranged on top of one another in layers in the form of a sandwich. The insertion of amino acids KLSRVD downstream of amino acid position 177, or downstream of amino acid position 200, did not interfere with the ability of this protein to assemble into these characteristic crystal-like particles. However, the insertion of amino acids PGEFLE downstream of amino acid position 144 resulted in no such particles being observed. It is likely that the VP7-144(P₂) trimer aggregates are less stable than those formed by the VP7-177(P₁) and VP7-200(P₁) trimers and are easily disrupted by resuspension in buffer and lead thus to random cellular debris being observed in the field. The insertion of the amino acids LQRPAR downstream of amino acid 177 in the top domain of the highly soluble VP7-144(P₂)-200(P₁) vector resulted in particles, ranging from 6-10µm in size, with a thick cookie-like appearance. The insertion of the FMDV antigenic peptide into the VP7-177(P₁) vector disrupted the ability of the VP7-177(P₁) vector protein to assemble into the characteristic hexagonal crystal-like particles, with a flat surface structure, resulting instead in the observation of uneven, distorted VP7-177(P₁)-FMDVepi particles (6-7µm).

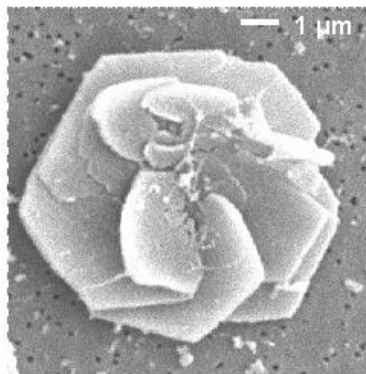
As these particulate VP7-177(P₁)-FMDVepi aggregates, as well as soluble VP7-177(P₁)-FMDVepi protein, were composed of trimers (2.3.5), it was investigated which one of these components was able to elicit a better insert-specific, neutralizing antibody immune response in guinea pigs.



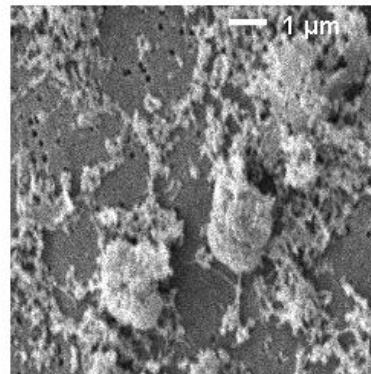
WT-VP7



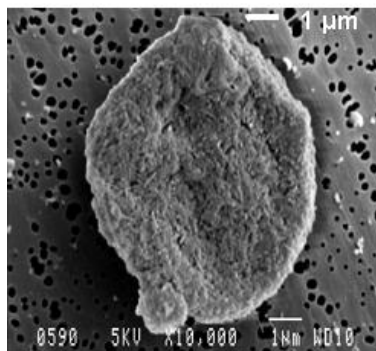
VP7-177(P₁)



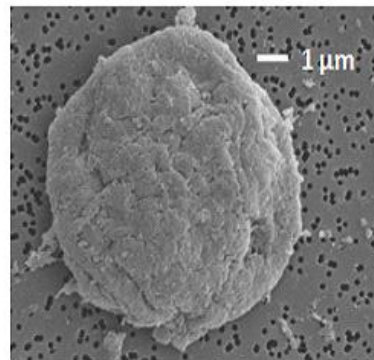
VP7-200(P₁)



VP7-144(P₂)



VP7-144(P₂)-177(P₃)-200(P₁)



VP7-177(P₁)-FMDVepi

Fig 2.9: Scanning electron microscopy (SEM) images of the particles characteristically observed in the particulate fractions of the WT-VP7, VP7-177(P₁), VP7-200(P₁), VP7-144(P₂), VP7-144(P₂)-177(P₃)-200(P₁) and VP7-177(P₁)-FMDVepi proteins. The images of the VP7-200(P₁) and VP7-144(P₂)-177(P₃)-200(P₁) particles are courtesy of Ms H. Kretzmann and Mr R. van Rensburg, respectively.

2.3.7 Assessment of the humeral immune response elicited by soluble and particulate VP7-FMDV fusion protein

The presence of soluble and particulate VP7-177(P₁)-FMDVepi protein raised the question which of these two components would induce the best insert-specific immune response. Previous studies had shown only a very weak immune response against a range of chimeric VP7 particles (unpublished results). A small pilot study in guinea pigs indicated that a strong FMDV-specific humeral immune response was elicited against the soluble trimer fraction of VP7-177(P₁)-FMDVepi and a relatively weak response against the particulate fraction (result not shown). In order to expand on these results, the following guinea pig trial was conducted. The VP7-177(P₁)-FMDVepi and VP7-177(P₁) proteins were harvested from Sf9 cells and their particulate and soluble fractions isolated from the appropriate sucrose gradient fractions as described in Materials and Methods (2.2.10). The isolated VP7 protein was quantified as described in the legend of Fig 2.10. The recovery of soluble VP7-177(P₁)-FMDVepi protein was approximately 6.5mg from 1x10⁸ Sf9 cells. Guinea pigs were inoculated with 40µg of soluble or particulate VP7-177(P₁)-FMDVepi protein in the presence of either Complete Freund's adjuvant (CFA) or Incomplete Freund's adjuvant (IFA) (2.2.11). The VP7-177(P₁) vector protein with no insert was included in this study as a negative control. Sera from the inoculated animals were tested for the presence of FMDV-specific antibodies via indirect ELISA and neutralizing antibodies via FMDV neutralizing assays (2.2.12). The results of this guinea pig trial are summarized in Table 2.4.

An insert-specific antibody response against the soluble VP7-177(P₁)-FMDVepi immunogen was detected 21 days after inoculation. This immune response was significantly enhanced by the booster inoculation and, at day 42, the average log₁₀ reciprocal ELISA titer, in animals inoculated with soluble VP7-177(P₁)-FMDVepi immunogen in the presence of CFA, was in the order of 3.7. The average log₁₀ reciprocal neutralizing antibody titer of these animals at 42 days post-inoculation was 2.6. The average log₁₀ reciprocal ELISA and neutralization titers, in guinea pigs inoculated with soluble VP7-177(P₁)-FMDVepi immunogen in the presence of IFA, were very similar, 3.4 and 2.6, respectively. The insert-specific antibody response in animals inoculated with particulate VP7-177(P₁)-FMDVepi immunogen in the presence of CFA was significantly lower with an average log₁₀ reciprocal ELISA titer of 2.2 and average log₁₀ reciprocal neutralization titer of 1.5. These differences were significant with *t*-test data analysis of the ELISA and virus neutralization titers indicating *p*-values of 0.0105 and 0.004, respectively. The response against the VP7-177(P₁) vector particles was considered a non-specific reaction as no FMDV-specific antibodies were expected to be elicited by this negative control.

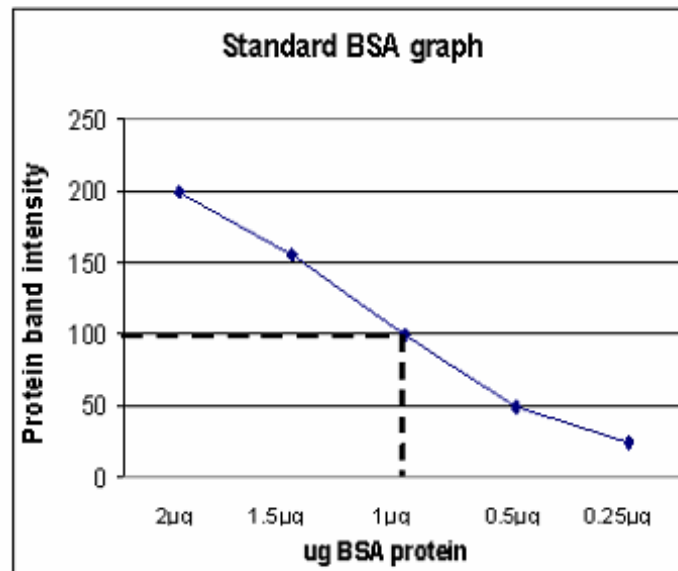
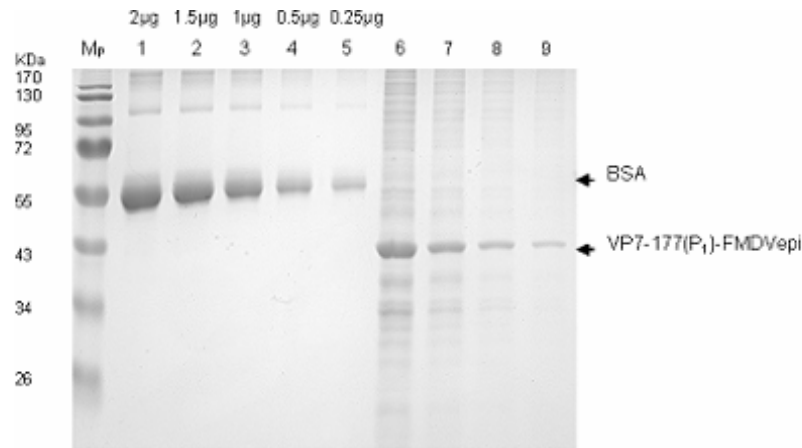


Fig 2.10: SDS-PAGE analysis to determine the concentration of the soluble VP7-177(P₁)-FMDVepi (42kDa) immunogen. Different volumes of the VP7-177(P₁)-FMDVepi protein sample, isolated from its respective sucrose gradient fractions, were electrophorized on an SDS-PAGE gel with known quantities of Bovine serum albumin (BSA) and stained with Coomassie Blue, as shown in the first panel. M_r is a PageRuler™ Prestained molecular size protein ladder (Fermentas). The relevant sizes are indicated in kDa. Lanes 1-5 contain 2µg, 1.5µg, 1µg, 0.5µg and 0.25µg BSA, respectively. Lanes 6-9 contain 1µl, 0.5µl, 0.25µl and 0.1µl of the VP7-177(P₁)-FMDVepi protein sample, respectively. Arrows on the side of the gel indicate the position of the BSA and VP7-177(P₁)-FMDVepi proteins. The EZQuant software program was used to determine the intensities of the protein bands on the PAGE gel and a standard BSA graph, correlating the quantities of BSA protein (in µg) with BSA protein band intensity was drawn (Panel 2). Using this graph, the quantity of VP7 protein was inferred from the intensity of the VP7 protein bands and the VP7 concentration calculated.

Table 2.4: Immune response in guinea pigs against soluble and particulate VP7-177(P₁)-FMDVepi protein

Antigen	Adjuvant And Dose	FMDV ELISA			FMDV Neutralization		
		Titres: Log ₁₀ reciprocal			Titres: Log ₁₀ reciprocal		
		21 days p.i	42 days p.i	Average ^(a)	21 days p.i	42 days p.i	Average ^(a)
VP7-177(P ₁)-FMDVepi (Soluble)	Complete Freund's (40µg)	1.9 2.2 4.1 3.8	3.5 3.2 4.1 4.1	3.7 (±0.45)	1.6 1.4 1.8 1.7	2.4 2.2 3.1 2.9	2.6 (±0.42)
	Incomplete Freund's (40µg)	2.8 2.8	3.5 3.2	3.4 (±0.21)	1.4 1.9	2.8 2.4	2.6 (±0.28)
VP7-177(P ₁)-FMDVepi (Particles)	Complete Freund's (40µg)	1.1 1.1 1.6	1.6 2.8 2.2	2.2 (±0.6)	1.2 1.2 1.2	1.2 1.8 1.5	1.5 (±0.28)
VP7-177(P ₁) vector	Complete Freund's (40µg)	1.2 1.3 1.3	1.3 1.3 1.3	1.3	ND ND ND	ND ND ND	ND

(a) - Standard deviation of the averages at 42 days in brackets
p.i - post-inoculation
ND - Not Determined

2.4 Discussion

Previous studies have explored the presentation of immunologically important epitopes on the surface of Bluetongue virus (BTV) core-like particles (Adler et al., 1998, Belyaev & Roy, 1992, Le Blois & Roy, 1993, Roy, 1995, Roy et al., 1997). When BTV major core proteins VP7 and VP3 are co-expressed in insect cells by means of recombinant baculovirus, these proteins spontaneously assemble into stable core-like particles or CLPs (French & Roy, 1990). An initial study indicated that a Hepatitis B virus (HBV)-VP7 fusion protein, consisting of a 48 amino acid HBV pre-S2 region fused to the N-terminal of BTV VP7, formed stable CLPs with the WT BTV VP3 protein only if the WT BTV VP7 protein was also expressed within the same cell (Belyaev & Roy, 1992). The insertion of foreign peptides into the Arg-Gly-Asp (RGD) (aa 168-170) and the Ala (aa145) sites within the BTV VP7 top domain, however, yielded stable CLPs, capable of eliciting an immune response, without WT VP7 co-expression (Adler et al., 1998, Roy, 1995, Roy et al., 1997). Peptides up to 39 amino acids in length have reportedly been inserted into BTV VP7 without affecting CLP stability (Roy et al., 1997). It is, however, likely that the insertion of longer peptides may affect the assembly of the capsid fusion proteins into stable particles, as reported elsewhere (Janssens et al., 2010, Peralta et al., 2009, Tanaka et al., 1995, Tissot et al., 2010).

Co-expression of major capsid proteins VP7 and VP3 of African horsesickness virus (AHSV) in insect cells has also been found to result in the formation of CLPs (Maree et al., 1998). The yield of these AHSV CLPs was, however, very low and hypothesized to be due to a large portion of the expressed VP7 protein forming the previously described crystal-like particles (Burroughs et al., 1994, Chuma et al., 1992) and thus not being available for CLP assembly. The VP7 particle formation is most likely due to the hydrophobic nature of the AHSV VP7 protein (Roy et al., 1991), resulting in a very insoluble protein (Maree, 2000). The crystal-like particles are composed of VP7 trimer molecules (Monastyrskaya et al., 1997) that are assembled into a highly ordered, symmetric structure, similar to way that VP7 trimers are arranged on the surface of the AHSV core particle (Burroughs et al., 1994).

AHSV-9 VP7 crystal-like particles were found to induce an immune response in mice and 80-100% of the immunized mice were protected against heterologous, virulent AHSV-7 challenge (Wade-Evans et al., 1997). These results prompted the suggestion that foreign antigenic peptides could be presented on the surface of such particles and elicit a protective immune response. Furthermore, the peptide-specific humoral and/or cellular immune response could be enhanced by the presentation of the peptide in a dense repetitive array on the surface of the VP7 crystal-like particle, similar to the strategy of virus-like particles (VLPs) and CLPs (Grgacic & Anderson, 2006, Mortellaro & Ricciardi-Castagnoli, 2011, Spohn & Bachmann, 2008). The effective display of antigenic peptides by AHSV VP7 would thus depend on the fusion proteins ability to form trimers, the assembly of these trimers into crystal-like particles and the presentation of the peptide in its native immunogenic conformation on the surface of such particles. In order to facilitate the insertion of foreign peptides into the AHSV VP7 protein, a number of VP7 vectors were constructed (Maree, 2000, Riley, 2003, Van Rensburg, 2004). The

effect of these 6-mer insertions into different sites within the VP7 top domain on the ability of the VP7 protein to form trimers, the assembly of these trimers into particles (solubility) and the morphology of these particles was investigated in this study.

Under the experimental conditions specified in this study, the relative amount of soluble wild type (WT) AHSV VP7 protein was determined to be less than 9% of the total quantity of VP7 expressed in recombinant baculovirus-infected insect cells. Using the same standardized experimental conditions, the solubility profiles of the different VP7 vector proteins were determined and compared to that of the WT VP7 protein. In the case of the VP7-144(P₂) and VP7-177(P₁) vector proteins, the insertion of the 6-mer peptides into the VP7 top domain increased protein solubility significantly. However, in the case of the VP7-144(P₁) and VP7-200(P₁) vector proteins, the 6-mer peptide insertions did little to increase VP7 protein solubility. In the case of the VP7-144(P₂)-177(P₃)-200(P₁) protein, solubility was reduced to a non-detectable level of soluble VP7 protein. These results indicated that VP7 protein solubility is influenced both by the inserted peptide as well as the insertion site in the VP7 top domain. Insertion of peptides P₁ or P₂ downstream of the VP7 amino acid 144 (Riley, 2003, Meyer, unpublished data) resulted in the VP7-144(P₁) and VP7-144(P₂) vector proteins having vastly differing solubility's, indicating the influence of the inserted peptide on VP7 protein solubility. Insertion of peptide P₁ downstream of VP7 amino acid 177 or 200 (Maree, 2000) resulted in the VP7-177(P₁) and VP7-200(P₁) vector proteins differing in solubility and indicated the influence of the insertion site. As the VP7 top domain is a determinant of VP7 protein solubility (Basak et al., 1996, Monastyrskaya et al., 1997), it is not surprising that peptide insertions that strengthen the impact of hydrophilic domains within the top domain would increase VP7 protein solubility, as in the case of the VP7-144(P₂) and VP7-177(P₁) proteins, while peptide insertions that destroy these hydrophilic domains would result in more insoluble chimeric VP7 proteins (VP7-144(P₁), VP7-200(P₁) and VP7-144(P₂)-177(P₃)-200(P₁)). The increased solubility of the VP7-177(P₁) vector protein when compared to the VP7-200(P₁) vector may be due to the insertion of the KLSRVD peptide into the highly flexible loop (aa175-180) (Basak et al., 1996). This flexible loop contains the RGD motif (Roy, 1996b) which is responsible for viral attachment to a cell surface receptor (Grimes et al., 1995, Tan et al., 2001). More optimal presentation of the inserted amino acids to the aqueous environment by this flexible loop may have increased the hydrophilic character of the VP7 top domain and resulted in increased VP7 protein solubility. Although the P₁ peptide (KLSRVD) contained a greater number of charged amino acid residues, the P₂ peptide (PGEFLE) was more optimally presented to the aqueous environment when inserted downstream of VP7 amino acid 144 resulting in the VP7-144(P₂) protein having a greater solubility when compared to that of the VP7-144(P₁) protein.

The VP7 solubility assays were conducted under the same standardized experimental conditions. The ratio of soluble versus aggregated or insoluble chimeric VP7 protein depends, however, on the concentration of VP7 protein in the cell and the ratio decreases rapidly from 24 to 72 hours post-infection (Mizrachi, 2008). This result implies that the VP7 protein solubility is not only determined by the VP7 construct but also by the time after

infection when the protein is harvested, the multiplicity of infection as well as other experimental factors. Although the solubility of the WT VP7 and VP7 vector proteins may differ under a different set of experimental conditions, conducting the solubility assays of the VP7 proteins under the same set of experimental conditions in this study allowed for a comparison of the solubility of the WT AHSV VP7 and VP7 vector proteins.

Additional 6-mer peptide insertions into the top domain of the VP7 vector proteins were also found to affect protein solubility. The insertion of the P₂ peptide downstream of amino acid 144 in the VP7-200(P₁) vector (Riley, 2003) converted this insoluble VP7 vector protein to the highly soluble VP7-144(P₂)-200(P₁) vector protein. The additional insertion of the P₃ peptide (LQRPAR) downstream of amino acid 177 in this soluble VP7-144(P₂)-200(P₁) vector (Van Rensburg, 2004), however, resulted in the formation of the highly insoluble VP7-144(P₂)-177(P₃)-200(P₁) protein. From the constructs investigated here it is, however, not yet possible to predict which constructs may yield the greatest number of insoluble VP7 protein crystal-like particles for the presentation of foreign peptides to the immune system.

As AHSV VP7 protein solubility was influenced by small 6-mer peptide insertions within this proteins top domain, the influence of these peptide insertions on VP7 trimer formation was also investigated. The ability of VP7 to form trimers is one of the criteria for the effective display of foreign peptides by this protein. As the N- and C-terminals of the VP7 protein, that together form the VP7 bottom domain, are important for the ability of VP7 monomers to fold correctly and form trimers (Hosamani et al., 2010, Monastyrskaya et al., 1997, Wang et al., 1994), it was not expected that the peptide insertions within the VP7 top domain would affect trimer formation. The soluble BTV VP7 protein has previously been found to have the sedimentation profile expected of a VP7 trimer following glycerol density sedimentation (Limn & Roy, 2003, Limn et al., 2000). The centrifugation conditions ensured size-based separation of the soluble VP7 trimers from soluble VP7 monomers and their sizes were confirmed by the inclusion of specific size marker proteins. Since VP7-144(P₂) was the protein with the largest soluble fraction, it was the first AHSV VP7 vector protein selected for trimer sedimentation analysis. The sedimentation analysis, using suitable sedimentation marker proteins, as well as a BTV VP7 control, indicated that VP7-144(P₂) had the sedimentation profile expected of a VP7 trimer molecule. The soluble components of the VP7-177(P₁), VP7-200(P₁) and VP7-144(P₂)-200(P₁) proteins were subsequently found to have sedimentation profiles comparable to that of the soluble VP7-144(P₂) trimer. These results indicated that the insertion of peptides P₁ or P₂ downstream of VP7 amino acids 144, 177 or 200 did not affect the ability of the resulting VP7-144(P₂), VP7-177(P₁), VP7-200(P₁) and VP7-144(P₂)-200(P₁) vector proteins to form trimers.

As the low density sucrose sedimentation strategy could only be used to determine whether soluble VP7 proteins formed trimers, an alternative protocol was employed to determine whether insoluble or aggregated AHSV VP7 protein was also composed of trimers. A modified version of the SDS polyacrylamide gel electrophoresis strategy was first used to assess whether the insoluble WT AHSV VP7 particles were composed of trimer molecules (Monastyrskaya et al., 1997). As WT AHSV VP7 trimers were shown to be stable in SDS at

temperatures up to at least 37°C, it was possible to distinguish between the VP7 trimer and VP7 monomer molecules based on their different rates of electrophoresis migration through a SDS polyacrylamide gel. The protein samples in this study were incubated at 25°C, rather than 37°C, prior to SDS polyacrylamide electrophoresis. Under these conditions it was confirmed, as expected, that the particulate WT AHSV VP7 as well as the insoluble or aggregated VP7-144(P₁), VP7-177(P₁) and VP7-144(P₂) vector proteins were composed of trimers. Although the soluble VP7-200(P₁) and VP7-144(P₂)-200(P₁) proteins were earlier found have the sedimentation profiles of trimer molecules, no trimers were detected using the modified SDS-PAGE trimer detection assay. This indicated that the VP7-200(P₁) and VP7-144(P₂)-200(P₁) trimers were not stable in the presence of SDS at room temperature and disassociated into monomers during SDS-PAGE. The partial disassociation of the VP7-144(P₁) and VP7-144(P₂) trimers during SDS-PAGE indicated that these trimers were also less stable than the WT VP7 and VP7-177(P₁) trimers in SDS at room temperature but more stable than the VP7-200(P₁) and VP7-144(P₂)-200(P₁) trimers.

The stability of the VP7 trimer molecules under strong denaturing conditions, as in the presence of SDS, seems to be strongly influenced by the site within the VP7 top domain into which the foreign peptide is inserted. This may be due to the spatial arrangement of the three copies of the insertion site on the top domain of the AHSV VP7 trimer molecule (Fig 1.5B). The three 177 sites are situated far apart on the surface of the VP7 trimer top domain. Similarly, the three 144 sites are also located far apart on the trimers top domain. Peptide insertions into either of these sites would thus be able to fold correctly without any steric interference and be presented on the surface of the VP7 trimer top domain without affecting trimer stability. The close proximity of the three 200 sites on the VP7 trimer top domain may have been responsible for the instability of the VP7-200(P₁) and VP7-144(P₂)-200(P₁) vector trimers in the presence of SDS. Steric hindrance between the closely positioned P₁ peptides may have resulted in a weakening of the VP7 monomeric interactions within the AHSV trimer molecule. This may indeed be the case as the VP7 top domain has also been suggested to play a role in trimer formation (Grimes et al., 1995).

(B)

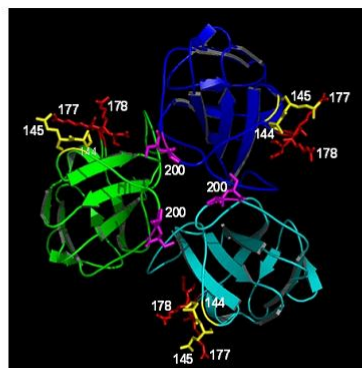


Fig 1.5(B) Computer generated model of the secondary structure of the top domain of an AHSV-VP7 trimer looking vertically onto the top of the trimer (Courtesy of Dr F. Maree). The spatial arrangement of the insertion sites 177-178 (Red), 144-145 (yellow) and 200-201 (pink) is indicated.

As the 6-mer peptide insertions into the VP7 top domain were found, in some cases, to affect VP7 protein solubility and trimer stability in SDS, the characteristic morphology of the WT AHSV VP7 crystal-like particles was also hypothesized to be affected (Burroughs et al., 1994, Chuma et al., 1992). To investigate the AHSV WT VP7, VP7-177(P₁), VP7-144(P₂) and VP7-200(P₁) particles was visualized under a scanning electron microscope. Insertion of the KLSRVD peptide downstream of amino acids 177 and 200 in the VP7 top domain did not interfere with the ability of the VP7 trimers to assemble into the 6-7µm hexagonal crystal-like particles also formed by the WT VP7 trimers. Insertion of the PGEFLE peptide downstream of amino acid 144, however, resulted in no such crystal-like particles being observed in the field. It is likely that the aggregates formed by the VP7-144(P₂) trimers were less stable than the WT VP7, VP7-177(P₁) and VP7-200(P₁) trimer aggregates and were solubilized by resuspension in phosphate buffered saline buffer.

Next the effect of inserting larger foreign peptides into the VP7 top domain on VP7 solubility, trimer formation and particle morphology was investigated. The first foreign peptide inserted into AHSV VP7 consisted of amino acids 129-164 of Foot-and-mouth disease virus (FMDV) VP1 protein, previously found to contain regions that were immunogenic (Bittle et al., 1982, DiMarchi et al., 1986, Doel et al., 1990, Morgan & Moore, 1990, Pfaff et al., 1982, Shao et al., 2011, Su et al., 2007). The resulting VP7-177(P₁)-FMDVepi fusion protein had a solubility of approximately 40%. This was an increase over the solubility of the VP7-177(P₁) vector protein (28%). The soluble and insoluble components of the VP7-177(P₁)-FMDVepi fusion protein were both shown to be composed of trimer molecules that were stable in SDS at room temperature. This stability was most likely due to the fact that the FMDV peptide was inserted into site 177(P₁), whose spatial position on the trimer top domain ensured that any inserted peptide was less likely to interfere with VP7-177(P₁) trimer stability. We also found the smooth, flat hexagonal appearance of the particles formed by the VP7-177(P₁) vector protein to be disrupted by the FMDV peptide insertion. Scanning Electron Microscopy (SEM) analysis revealed the VP7-177(P₁)-FMDVepi fusion protein particles (6-7µm) to be round with a rough, textured surface.

Although the effective display of foreign peptides by AHSV VP7 was initially thought to be dependent on the assembly of VP7 trimers into insoluble particles, some preliminary studies indicated that chimeric VP7 particles were only capable of eliciting a weak humeral immune response against the peptide being presented (unpublished results). As both the particulate and soluble components of the VP7-177(P₁)-FMDVepi fusion protein were composed of trimers, the question was raised whether there was any difference in the immunogenicity of the FMDV peptide when presented by soluble or particulate VP7 trimers. The FMDV peptide contained B-cell linear epitope regions aa141-160, aa135-164, aa131-134 and T-cell epitope region aa141-160 (Su et al., 2007) and has previously elicited protective neutralizing antibodies in guinea pigs and cattle (Bittle et al., 1982, DiMarchi et al., 1986, Doel et al., 1990, Morgan & Moore, 1990, Pfaff et al., 1982, Shao et al., 2011). The presentation of this peptide fused to carrier proteins, such as the *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) or the swine immunoglobulin G heavy chain constant region (sclgG), elicited humeral and cellular immune responses and protection in target host animals (Shao et al., 2011, Su et al., 2007). It was thus

hypothesized the presentation of the FMDV VP1 peptide (aa129-164) by the AHSV VP7 protein may also elicit a FMDV neutralizing, humeral immune response in guinea pigs.

Large quantities of both the particulate and soluble VP7-177(P₁)-FMDVepi were harvested for the small animal trial. 1×10^8 baculovirus-infected insect cells yielded approximately 6.5mg of soluble VP7-177(P₁)-FMDVepi protein indicating one of the advantages of the baculovirus expression system. The small animal trial indicated that not only was the FMDV VP1 peptide able to elicit FMDV-specific, neutralizing antibodies but that the antibody titers elicited against the soluble VP7-177(P₁)-FMDVepi trimers were, on average, as much as ten times higher than those elicited against the particulate VP7-177(P₁)-FMDVepi fusion protein. It is likely that soluble trimers display a larger number of peptide copies to the immune system than trimers in an aggregated form and, due to this increased surface area, an enhanced immune response was elicited. This result focused our attention on the use of soluble VP7 trimer molecules as a novel antigen display system. It was also found that inoculation of guinea pigs with soluble VP7-177(P₁)-FMDVepi trimers in the presence of Complete Freund's adjuvant (CFA) did not significantly enhance FMDV-specific, neutralizing immune response elicited when compared to the same antigen in Incomplete Freund's adjuvant (IFA). IFA, lacking the *Mycobacterium tuberculosis* component (Oscherwitz et al., 2006), may thus be used as an alternative to CFA in future animal studies in order to minimize the adverse localized reaction of animals to the *Mycobacterium tuberculosis* component.

In order to increase the soluble VP7 trimer component, one strategy that was explored was the mutation of a specific amino acid within the bottom domain of the AHSV-9 VP7 protein (Meyer, 2002). The substitution of a non-polar, uncharged leucine residue in position 345 of AHSV VP7 with a polar, positively charged arginine residue, the BTV equivalent, was suggested to increase AHSV VP7 protein solubility (Monastyrskaya et al., 1997). This increase in protein solubility was hypothesized to be due to an interaction of the hydrophilic arginine residue with the hydrophilic region harboring the RGD motif (aa168-176) (Basak et al., 1996) on an adjoining VP7 monomer. A preliminary result with WT AHSV VP7 and VP7-177(P₁) proteins containing this amino acid mutation within their C-terminals indicated that this mutation resulted in a significant increase in protein solubility (Meyer, 2002). This result was confirmed in this study under standardized experimental conditions and indicated that the solubility of the AHSV WT VP7 protein could be increased to 48% by mutating the leucine residue in position 345 to an arginine residue. Similarly the solubility of the VP7-177(P₁) vector protein increased to 40% following the same mutation within this protein's C-terminal. Using a modified SDS-PAGE strategy it was also confirmed that, although the C terminal of VP7 plays a major role in trimer formation (Hosamani et al., 2010), this specific amino acid mutation did not affect trimer formation. These trimers were also as stable in the presence of SDS at room temperature as the parent WT VP7 and VP7-177(P₁) proteins. As this C-terminal mutation increased the solubility of these VP7 proteins without affecting trimer formation and stability, it may be considered for inclusion into future VP7 vectors to increase protein solubility.

Chapter 3

Expanding the range of peptides inserted into VP7 and enhancing the immune response against these VP7 fusion proteins

3.1 Introduction

The effective display of antigenic peptides by African horsesickness viral protein 7 depends on firstly, the ability of the VP7 fusion protein to form trimers, secondly the solubility of these VP7 trimers and thirdly the ability of the peptide to fold correctly and be presented in its native immunogenic conformation on the surface of the VP7 trimer. In the previous chapter it was determined that the peptide insertion site within the VP7 top domain, as well as the character of the inserted peptide, influence the above mentioned display criteria. The insertion of a well characterised antigenic peptide from the VP1 protein of Foot-and-mouth disease virus (FMDV) (Bittle et al., 1982, Morgan & Moore, 1990, Pfaff et al., 1982, Shao et al., 2011, Zamorano et al., 1998) (aa129-164) into site 177(P₁) of the VP7 top domain increased the solubility of the VP7-177(P₁) vector protein, resulted in the formation of stable trimers and allowed for the correct folding of this peptide into its native immunogenic conformation. Here the range of foreign peptides inserted into the VP7 top domain is expanded by investigating the effect of these insertions on VP7 protein solubility, trimer formation and the ability of the peptide to fold correctly.

Peptides, derived from the outer capsid proteins VP1 of FMDV and VP2 of AHSV, as well as the envelope protein gp41 of HIV-1, were inserted into different sites within the AHSV-9 VP7 top domain in this and previous studies (Table 2.2). The insertion of the same peptide into different sites would indicate the effect of the insertion site on the VP7 display criteria mentioned above whereas the insertion of different peptides into the same site would indicate the influence of the inserted peptide. A diagrammatic representation of the FMDV, AHSV and HIV peptides that were inserted into different sites within the AHSV-9 VP7 top domain is shown in Figures 3.1 and 3.2. These peptides all contained previously described immunogenic epitopes or epitope-equivalent sequences. The FMDV peptide (aa129-164) is well characterised and contained a number of B-cell and T-cell epitopes (Aggarwal & Barnett, 2002, Bittle et al., 1982, Morgan & Moore, 1990, Pfaff et al., 1982, Robert, 2003, Shao et al., 2011, Zamorano et al., 1998). Epitopes 'A' (aa321-339) and 'B' (aa377-400) of the VP2 capsid protein of AHSV serotype 4 have previously been found to be neutralizing (Martinez-Torrecuadrada et al., 2001) and were inserted into VP7 individually, as well as in combination with each other on larger AHSV-4 VP2 peptides, up to 150 amino acids in length (Fig 3.3). A strong linear AHSV-9 VP2 epitope 'B' (aa377-400), identified within a large hydrophilic domain between amino acids 369 and 403 (Venter et al., 2000), was also inserted into VP7. The inserted HIV-1 peptides contained the HIV-1 subtype B broadly neutralizing ELDKWA (Dong et al., 2001, Muster et al., 1993) or RILAVEYLKD (Chen & Dierich, 1998, Dong et al., 2001) epitopes, with or without the amino

acids flanking these epitopes within the subtype B gp41 protein. As HIV-1 subtype C is the most prevalent HIV-1 subtype in sub-Saharan Africa (McCutchan, 2006), HIV-1 subtype C sequences equivalent to these subtype B epitopes, were also inserted with or with their respective subtype C flanking amino acids. As the amino acid sequence of the regions flanking the epitopes/epitope-equivalent domains differed, these flanking regions were designated f_1 - f_8 (Fig 3.2). A schematic representation of the location of the subtype B epitopes, and the corresponding subtype C epitope-equivalent domains, on the gp41 protein is shown in Fig 5.1. Flanking amino acids were added in order to present the epitopes, or epitope-equivalent sequence, as larger peptides on the surface of the VP7 trimer thus enhancing their display to the immune system. The subtype C LGLWGCSGKIIC (aa 574-585), partially overlapping the equivalent HIV-1 subtype B GCSGKLICTT epitope (aa 590-599) (Cotropia et al., 1996, Viveros et al., 2000) was inserted with a number of highly hydrophilic arginine residues on its N-and C-terminals. This strategy was proposed to increase VP7 fusion protein solubility as a greater number of surface exposed, hydrophilic amino acid residues have been shown to enhance protein solubility (Bianchi et al., 1994, Mosavi & Peng, 2003).

The guinea pig trial in chapter 2 indicated that soluble VP7-177(P₁)-FMDVepi trimers provide an advantage over aggregated trimers in immune display. As it not yet possible to predict the solubility of the VP7 fusion protein by selecting a specific peptide or insertion site, extrinsic strategies of improving the solubility of VP7 fusion trimers were investigated. Factors that influence protein solubility include pH, temperature and salt concentration. Different solvent conditions, therefore, need to be investigated to identify the optimum conditions for high solubility of the protein of interest (Howe, 2004, Jancarik et al., 2004). Various chemical treatments also increase protein solubility, more specifically treatment with the anionic detergent Na⁺*N*-Lauroylsarcosine (Sarkosyl) (Frankel et al., 1990) and amino acid L-Arginine (Tsumoto et al., 2004). These chemicals reverse or prevent protein aggregation (Frankel, 1991, McNally et al., 1991, Tsumoto et al., 2005) thus solubilizing protein aggregates without negatively affecting the native form and function of the protein and are currently being used in a wide spectrum of studies (Grathwohl et al., 1996, Ishibashi et al., 2005, Skoglund et al., 2008, Tsumoto et al., 2010). One of the hypotheses for protein insolubility is the accumulation of partially folded forms of protein which then aggregate via hydrophobic interactions (Mitraki, 2010). The sodium salt of the alkyl anionic detergent *N*-laurylsarcosine (sarkosyl) has been shown to solubilize such protein aggregates, or prevent their formation, by absorbing to the partially folded proteins and interfering with their hydrophobic interactions (Frankel, 1991, McNally et al., 1991). Even correctly folded proteins, that have a tendency to aggregate, maybe solubilized with low concentrations of sarkosyl without losing their functional activity (Frangioni & Neel, 1993, Frankel et al., 1990, Tan & Spudich, 1991). Sarkosyl is, however, a detergent and may result in the denaturation of protein if used at higher concentrations. Although the integrity of the AHSV VP7 trimer molecule would most likely not be compromised following treatment with sarkosyl due to trimer stability in the presence of the stronger SDS detergent (Chapter 2), the conformation of non-linear epitopes presented on the surface of the trimer may be affected. Detergents also have a tendency to bind to proteins with a high affinity and are difficult to remove (Kudou et al., 2011).

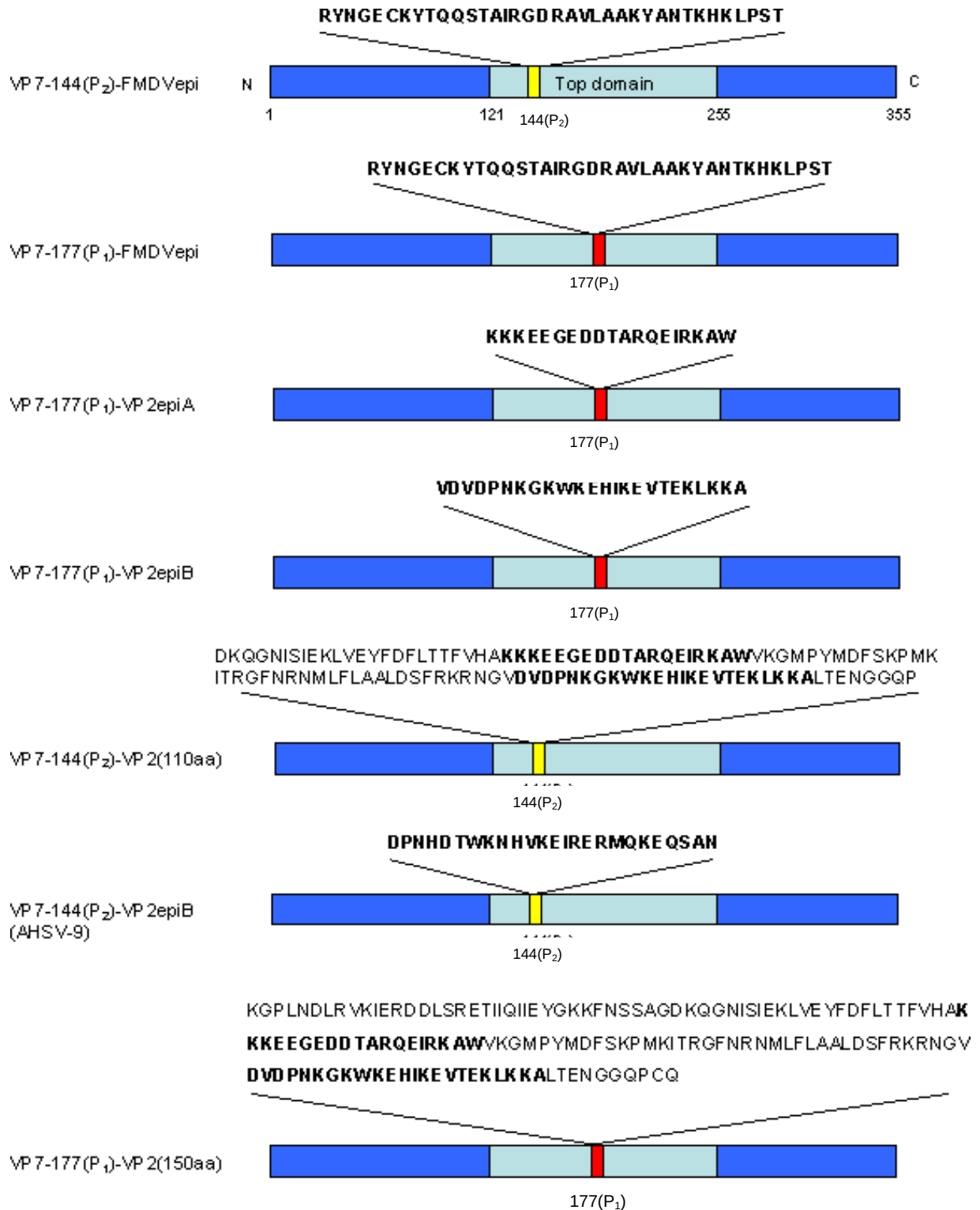


Fig 3.1: A schematic representation of the VP7-FMDV and VP7-AHSV fusion proteins characterized in this study. Antigenic peptides from FMDV VP1 and the AHSV VP2 outer capsid proteins were inserted into sites 177(P₁) or 144(P₂) within the VP7 vector top domain (amino acids 121-255) as indicated. Epitope amino acid sequences are highlighted in bold. Additional construct information is given in Table 2.2.

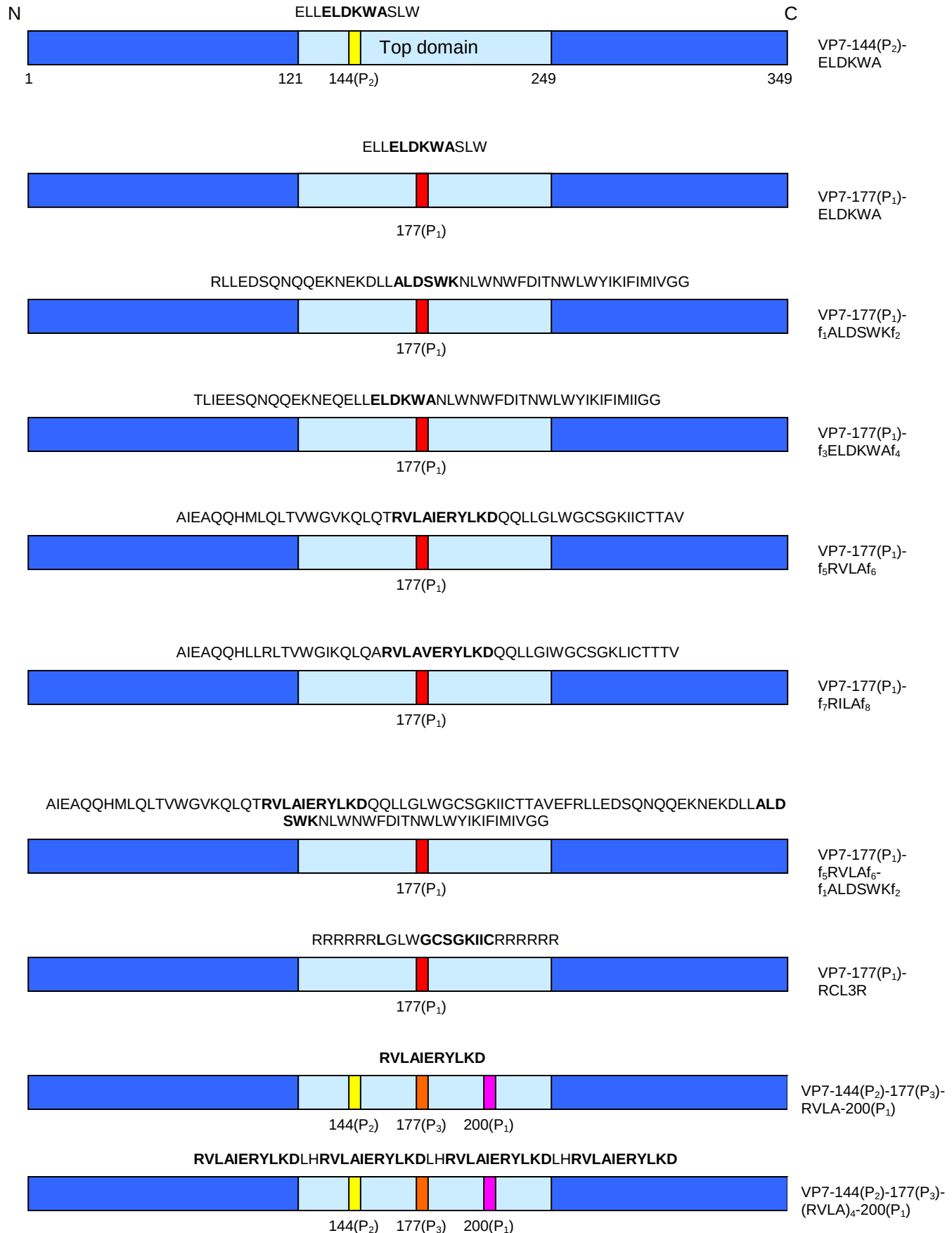


Fig 3.2: A schematic representation of the VP7-HIV fusion proteins used in this study. Antigenic peptides from gp41 protein of HIV-1 subtypes B and C were inserted into sites 144(P₂), 177(P₁) or 177(P₃), within the VP7 vector top domain (aa121-255), as indicated. Epitope amino acid sequences are highlighted in bold. f indicates amino acids flanking the epitopes. Additional construct information is given in Table 2.2.

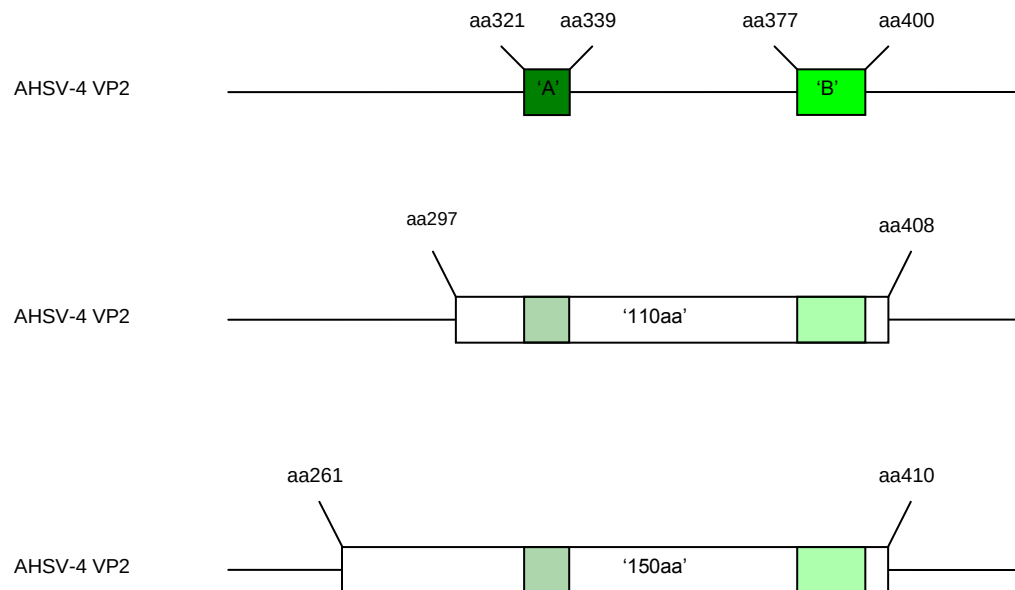


Fig 3.3: A schematic representation of antigenic AHSV-4 VP2 regions inserted into the AHSV-9 VP7 top domain. The neutralizing epitopes 'A' (aa321-339) and 'B' (aa377-400) (Martinez-Torrecuadrada et al., 2001) that were inserted are indicated in green. The larger inserted domains, containing both these epitopes and either 110 or 150 amino acids in length, are also indicated.

Like detergents, the amino acid L-arginine is also able to solubilize aggregates of various proteins (Golovanov et al., 2004, Maity et al., 2009, Shubenok et al., 2009, Tsumoto et al., 2010, Tsumoto et al., 2003, Umetsu et al., 2005). Although high concentrations of L-arginine (0.5M-2M) are required for effective aggregate solubilization (Arakawa et al., 2007, Tsumoto et al., 2004), this amino acid need not be removed following treatment of the protein. Recent studies indicate that arginine's guanidinium group interacts with hydrophobic surfaces or aromatic residues effectively shielding these regions and preventing protein-protein aggregative interactions, resulting in an increase in protein solubility (Ito et al., 2011, Li et al., 2010). Unlike detergents, L-arginine does not denature the target protein at or below room temperature (Ishibashi et al., 2005), making it suitable for the solubilization of VP7 trimer aggregates presenting conformational epitopes, and even stabilizes the protein during storage (Tsumoto et al., 2004). L-arginine has also been shown to stimulate the immune response in immunized animals (Tayade et al., 2006).

In this study the effect of sarkosyl and L-Arginine treatment on the VP7 fusion protein solubility and the immunogenicity of the inserted foreign peptide will be investigated. The VP7 antigen dose required for an

optimum insert-specific, neutralizing antibody immune response will be also be determined, as well as which adjuvant enables the induction of a good humeral immune response.

3.2 Materials and Methods

3.2.1 Modified AHSV-9 VP7 constructs

The VP7-177(P₁)-150aa, VP7-177(P₁)-f₃ELDKWaf₄, VP7-177(P₁)-f₇RILAf₈ and VP7-177(P₁)-RCL3R constructs were designed for expression by baculovirus recombinants as described in the Bac-to-Bac[®] baculovirus expression system manual (Invitrogen). Briefly, the AHSV-4 VP2 nucleotide sequence, encoding an antigenic domain (amino acids 261-410), was amplified from a TOPO[®] XL-AHSV-VP2 plasmid template using the primers given in Table 2.1. Also, the HIV-1 subtype B gp41 nucleotide sequences, encoding the ELDKWA (aa637-684) and RILAVERYLKD (aa 551-601) epitope sequences with their flanking amino acids, were amplified from a pHXB2-HIV1subtypeB-gp160 plasmid template (provided by the National Institute of Virology) using the primers indicated in Table 2.1. Similarly, the HIV-1 subtype C sequence, encoding the immunodominant domain (amino acids 574-585), was amplified from a pHXB2-HIV1subtypeC-gp160 plasmid template (Rutkowska, 2002) with the primers indicated in Table 2.1, resulting in an additional 6 arginine residues flanking the N- and C-terminals of the resulting peptide. The resulting PCR amplicons were subsequently cloned into the VP7-177(P₁) vector utilizing the *Hind*III and *Sal*I sites.

The high fidelity TaKaRa Ex Taq[™] PCR kit (TAKARA BIO INC.) was used to perform all the PCR amplifications. The 50µl PCR reactions were set up according to the manufacturer's instructions using a final concentration of 0.5µM for each primer. The cycling conditions for the amplification of the AHSV-4 VP2 sequence: 1 cycle of 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 64°C for 45 sec and 72°C for 2.5 min followed by 1 cycle of 72°C for 10 min. The cycling conditions for the amplification of the HIV-1 subtype B sequence were as follows: 1 cycle of 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C/72°C for 1 min and 72°C for 2 min followed by 1 cycle of 72°C for 10 min. ELDKWA primers annealed to target sequences at 55°C while the RILAVERYLKD primers annealed at 72°C. The cycling conditions for the amplification of the HIV-1 subtype C gp41 immunodominant domain sequence was as follows: 5 cycles of 94°C for 1 min, 37°C for 45 sec, 72°C for 1 min followed by 25 cycles of 94°C for 1 min, 61°C for 45 sec, 72°C for 1 min. All amplicons and constructs were verified by chain termination sequencing (Applied biosystems).

3.2.2 Expression and characterization of VP7 fusion proteins

Recombinant baculoviruses were used to infect *Spodoptera frugiperda* cells, as described previously (2.2.4). The expressed VP7 fusion proteins were harvested 38, 48 or 72 hours post-infection. Cell pellets were resuspended in either 0.01M STE buffer or Lysis buffer (0.01M STE with 0.5% Nonidet[®]P40 (Roche), as

indicated in the specific experiment. Solubility of the VP7 fusion analysed via sucrose density sedimentation analysis (2.2.5). The protein content of each sucrose fraction was ascertained by SDS-polyacrylamide gel electrophoresis (2.2.6). The identity of the VP7 fusion proteins was confirmed via immunoblot analysis with a VP7-specific primary antibody (2.2.7) and quantification of the relative amount of soluble VP7 fusion protein was performed from the Coomassie blue stained PAGE gels, and/or the immunoblot membranes, by utilising the EZQuant software package (2.2.8). Trimer formation by the VP7 fusion proteins was assessed by means of a modified SDS-PAGE protocol, where protein samples were incubated at room temperature in the presence of SDS prior to electrophoresis (2.2.6).

3.2.3 Strategies to increase the yield of soluble VP7 fusion protein

Recombinant baculovirus-infected Sf9 cells were harvested 72 hours post-infection, as described in 2.2.4. The cell pellet was subsequently resuspended in 1000µl Lysis buffer (0.01M STE with 0.5% Nonidet[®] P40 (Roche) and incubated for 30 min on ice. If required in treatment of cellular lysate, the desired concentrations of L-Arginine amino acid (Merck) or *N*-lauroylsarcosine (Sarkosyl) (Merck) were added at this stage. Cellular lysate containing the desired protein was obtained following homogenization of the cell suspension by 20 strokes with a 1ml dounce homogenizer (Wheaton). Solubility of the VP7 fusion analysed via sucrose density sedimentation analysis (2.2.5). The protein content of each sucrose fraction was ascertained by SDS-polyacrylamide gel electrophoresis (2.2.6).

Treatment of only the particulate component of the VP7 fusion proteins with arginine or sarkosyl was performed following the centrifugation of the cellular lysate, containing VP7 fusion protein, through a 40% sucrose cushion at 27342 xg (15000rpm) for 20min at 4°C. The supernatant was discarded and the particulate component of the VP7 fusion protein, which had centrifuged through into the pellet, was resuspended in 1xPBS buffer (usually 1/10 of the lysis buffer volume in which the cells were originally resuspended). The particulate VP7 fusion protein was subsequently treated with the desired concentrations of the amino acid L-Arginine (Merck) or ionic detergent Na⁺*N*-lauroylsarcosine (Sarkosyl) (Merck) and incubated for 30 min at 25°C. Treated protein samples were either centrifuged at 27342 xg (15000rpm) for 20min at 4°C to remove residual insoluble proteins or through 50-70% sucrose density gradients at 109368 xg (30000rpm) for 18 hours at 4°C (2.2.5) to ascertain protein solubility. The protein content of the sucrose fractions, pellets and/or supernatants was analyzed by 12% SDS-PAGE (2.2.6). Trimer stability of the treated VP7 fusion proteins was assessed by means of a modified SDS-PAGE protocol, where protein samples were incubated at room temperature in the presence of SDS prior to electrophoresis (2.2.6).

3.2.4 Antigen preparation for the animal trials

Arginine or sarkosyl-solubilized VP7-177(P₁)-FMDVepi, VP7-177(P₁)-VP2epiA, VP7-177(P₁)-VP2epiB, VP7-144(P₂)-VP2(110aa) and VP7-177(P₁)-VP2(150aa) fusion proteins, as well as the VP7-144(P₁) vector protein, were prepared as follows:

Sf9 cells, infected with the appropriate recombinant baculovirus, were harvested 72 hours post-infection. Cellular lysates were prepared, as previously described (2.2.10), and the particulate protein fraction collected via centrifugation through a 40% sucrose cushion at 40572 xg (15000rpm) for 20min in a Beckman SW28 rotor at 4°C. The proteins were resuspended in sterile 1xPBS buffer, treated with either 1M L-arginine (Merck) or 0.5% Na⁺N-lauroylsarcosine (Sarkosyl) (Merck), incubated for 30 min at 25°C and centrifuged at 40572 xg (15000rpm) for 20 min at 4° to remove any residual insoluble proteins. The soluble arginine-treated samples were not subjected to any further purification procedures. The sarkosyl-solubilized VP7-177(P₁)-FMDVepi protein was dialyzed in SnakeSkinTM dialysis membrane (Pierce) against sterile distilled water to remove excess sarkosyl detergent. The arginine and sarkosyl-solubilized protein samples were freeze-dried in a Freezone 2.5 freeze-drier (Labconco) for 72 hours and reconstituted in sterile water. The proteins were recovered with no detectable loss of soluble protein. The quantity of VP7 protein was calculated from a standard curve drawn using known quantities of bovine serum albumin (BSA), as described previously (2.2.10).

3.2.5 Immunization protocols for animal trials

The guinea pig trials were conducted under the strict supervision of the Onderstepoort Animal Ethics Committee according to national standards. The studies were performed at the Transboundary Animal Diseases Program (TADP) of the Onderstepoort Veterinary Institute (OVI). Separate vaccines were formulated by combining the VP7 antigen solutions with an equal volume of one of the following adjuvants: Complete Freund's, an Al(OH)₃/Saponin combination or as double oil emulsions with MontanideTM ISA 206B (Seppic, Paris). Female guinea pigs (500-600g), acquired from Onderstepoort Biological Products, were inoculated subcutaneously with between 20µg and 80µg of VP7 antigen in a final volume of 500µl, which included 250µl of the adjuvant. Boost inoculations, with the same antigen dose and adjuvant mixtures, were administered three weeks after the primary inoculation. Guinea pigs were bled prior to immunization (pre-bleed), before the boost inoculation at 21 days and three weeks after the boost inoculation on day 42 of the animal trial.

3.2.6 Analysis of FMDV and AHSV antibodies by ELISA and virus neutralization

The FMDV ELISA and virus neutralization assays were performed at the Transboundary Animal Diseases Program (TADP) of the Onderstepoort Veterinary Institute (OVI) as described previously (2.2.12). Sera from the experimental animals were tested for the presence of anti-FMDV antibodies via indirect ELISA. Titration

endpoints are expressed as the $\log_{10}$ reciprocal of the highest dilution at which the signal obtained is twice the signal of the negative control. Under these conditions pre-bleed sera and negative controls had ELISA titers of 1.3 or less. The presence of neutralizing FMDV antibodies in the sera of VP7-177(P₁)-FMDVepi inoculated animals was detected via cross-neutralization assays in micro-titer plates on IB-RS-2 cells as described in the OIE Manual of Standards (2009). The titers were expressed as the $\log_{10}$ reciprocal of the last dilution of serum to neutralize 100 TCID₅₀ in 50% of the wells (Rweyemamu et al., 1978). All the data was statistically analyzed by a two sample t-test assuming unequal variance.

The AHSV ELISA and virus neutralization assays were performed by Dr. Q. Meyer and Mr P. Wege, respectively. Antisera, from animals inoculated with the VP7-177(P₁)-VP2epiA, VP7-177(P₁)-VP2epiB, VP7-144(P₂)-VP2(110aa) and VP7-177(P₁)-VP2(150aa) antigens, were tested for the presence of AHSV-4 epitope 'A' or AHSV-4 epitope 'B' antibodies via indirect ELISA. The ELISA plates were coated with AHSV-4 VP2epiA-BSA, a conjugate of AHSV-4 VP2 epitope A peptide (KKKEEGEDDTARQEIRKAW) and BSA in a 5:1 ratio (GL Biochem (Shanghai) Ltd) or AHSV-4 VP2epiB-BSA, a conjugate of AHSV-4 VP2 epitope B peptide (VDVDPNKGKWKKEHIKEVTEKLKKA) and BSA in a 5:1 ratio (GL Biochem (Shanghai) Ltd). The titration endpoints calculated to be the dilution at which the signal obtained is above the signal of the negative control. The presence of neutralizing AHSV-4 antibodies in the sera of inoculated animals was detected via plaque-reducing assays on Vero cells. The neutralizing antibody titers were expressed as the $\log_{10}$ reciprocal of the serum dilution causing a 50% reduction in the number of plaques. All the data was statistically analyzed by a two sample t-test assuming unequal variance. An antiserum used by Onderstepoort Veterinary Institute for their neutralization tests, Antiserum 32/62, was mixed with the AHSV-4 and used as a positive control.

3.3 Results

3.3.1 Effect of insertion site and peptide on VP7 solubility

To further investigate the determinants of VP7 fusion protein solubility, foreign peptides, derived from the outer capsid proteins VP1 of FMDV and VP2 of AHSV as well as the envelope protein gp41 of HIV-1, were inserted into different sites within the AHSV-9 VP7 top domain. The solubility of the resulting VP7 fusion proteins was subsequently analysed.

3.3.1.1 Solubility of VP7-FMDV-VP1 fusion proteins

To determine the effect of the insertion site on VP7 fusion protein solubility, the FMDV VP1 peptide (aa129-164), previously inserted into site 177(P₁) (2.3.5), was also inserted into site 144(P₂) (Table 2.2). The solubility of the

VP7-177(P₁)-FMDVepi and VP7-144(P₂)-FMDVepi fusion proteins was assessed at 72 hours post-infection under standardized experimental conditions (3.2.2) and the resulting SDS-PAGE gels are shown in Fig 3.4.

As previously, the soluble VP7-177(P₁)-FMDVepi protein, co-sedimenting with the soluble cellular and viral proteins in sucrose fractions 7 and 8, accounted for approximately 40% of the total VP7 protein expressed. In contrast, only 15% of the VP7-144(P₂)-FMDVepi protein was considered soluble, with the bulk of this protein sedimenting into the pellet of the sucrose gradient (Table 3.1). The range of VP7-144(P₂)-FMDVepi aggregates of various sizes distributed throughout the sucrose gradient suggested that this protein has a tendency to aggregate. Thus the increased protein aggregation following insertion of the FMD peptide into site 144(P₂) may have led to the low VP7-144(P₂)-FMDVepi protein solubility observed. Next, the influence of the inserted peptide on VP7 solubility was investigated by analysing the solubility of various VP7-AHSV-VP2 fusion proteins.

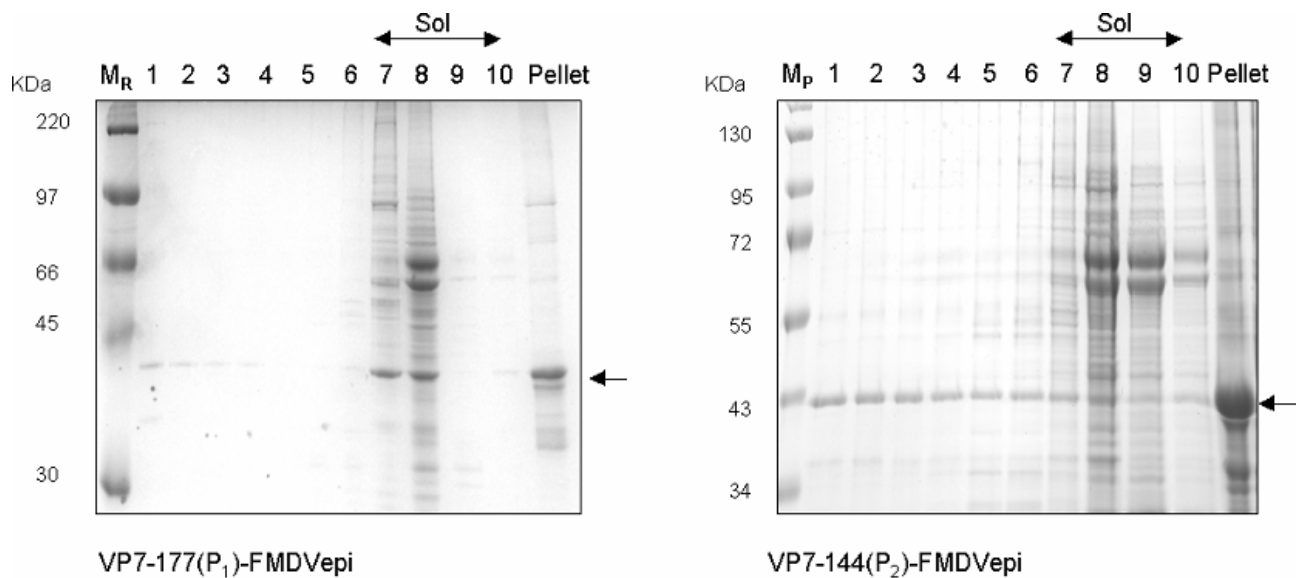


Fig 3.4: SDS-PAGE analyses of the fractions obtained following sucrose gradient sedimentation of VP7 fusion proteins VP7-177(P₁)-FMDVepi (42KDa) and VP7-144(P₂)-FMDVepi (42KDa). Cellular lysates were layered onto 50-70% sucrose gradients and centrifuged in a Beckman SW55Ti rotor for 18 h at 30000rpm. Fractions were collected from the bottom of the tube and equal quantities of each fraction and pellet analyzed by SDS-PAGE and the proteins visualized by Coomassie blue staining. Lanes 1-10 contain fractions 1-10 of the sucrose gradient, respectively. Double-sided arrows at the top of the gel indicate the soluble fractions of the gradient. M_R and M_P are the Rainbow™ (Amersham Pharmacia) and PageRuler™ (Fermentas) prestained molecular size protein ladders, respectively. The relevant sizes are indicated in KDa. The arrow on the side indicates the position of a unique protein, corresponding to the expected size of the VP7 fusion protein.

3.3.1.2 Solubility and trimer formation of the VP7-AHSV-VP2 fusion proteins

In order to determine the effect of the inserted peptide on VP7 fusion protein solubility, AHSV-4 VP2 epitopes 'A' (aa321-339) and 'B' (aa377-400) (Martinez-Torrecuadrada et al., 2001), as well as a larger AHSV-4 VP2 150aa peptide (aa261-410) (150aa) containing both these epitopes (Fig 3.3), were each inserted individually into the site 177(P₁) (Smalle, 2006) (Current Study). In addition, the AHSV-9 VP2 'B' epitope (aa377-400) (Venter et al., 2000) and a 110aa AHSV-4 VP2 peptide (aa298-408), containing both the AHSV-4 'A' and 'B' epitopes, were also inserted individually into site 144(P₂) (Riley, 2003) (Meyer, personal communication). The solubility of the resulting VP7-AHSV-VP2 fusion proteins (Table 2.2) was assessed at 72 hours post-infection as described in Materials and Methods (3.2.2) under standardized experimental conditions and the resulting SDS-PAGE gels shown in Fig 3.5. The solubility of each VP7-VP2 fusion protein is indicated in Table 3.1. The effect of the AHSV VP2 peptide insertions on the ability of the VP7 protein to form trimers was assessed via a modified SDS-PAGE protocol, described in 3.2.2 and the legend to Fig 3.5. The identity of the VP7 bands was established by immunoblotting with VP7-specific antibodies as indicated in the legends of the figures.

The insertion of the 19-mer AHSV-4 VP2 'A' epitope into site 177(P₁) resulted in 53% of the expressed VP7-177(P₁)-VP2epiA fusion protein sedimenting in soluble sucrose fractions 8-10. We had previously shown that the insertion of the 36-mer FMDV peptide into the same site resulted in the VP7-177(P₁)-FMDVepi protein having a solubility of approximately 40% (3.3.1.1). In contrast, the insertion of the 24-mer AHSV-4 VP2 'B' epitope into site 177(P₁) resulted in only 8% of VP7-177(P₁)-VP2epiB sedimenting in the soluble fractions with the majority located in the pellet of the sucrose gradient. The solubility of the VP7-177(P₁)-VP2(150aa), presenting a 150-mer AHSV-4 VP2 peptide containing both epitopes 'A' and 'B', was also only 5%. These results indicated that the peptide inserted into a specific site within the top domain had an influence on the solubility of the resulting VP7 protein. This was confirmed when insertion of the AHSV-9 VP2 epitope 'B' into site 144(P₂) within the VP7 top domain resulted in the VP7-144(P₂)-VP2epiB protein having a solubility of approximately 58% while the solubility of the VP7-144(P₂)-VP2(110aa) protein, presenting an 110-mer insert containing the both AHSV-4 VP2 epitopes 'A' and 'B', was only 8%. The VP7-144(P₂)-FMDVepi protein had also previously been shown to have a low solubility (3.3.1.1).

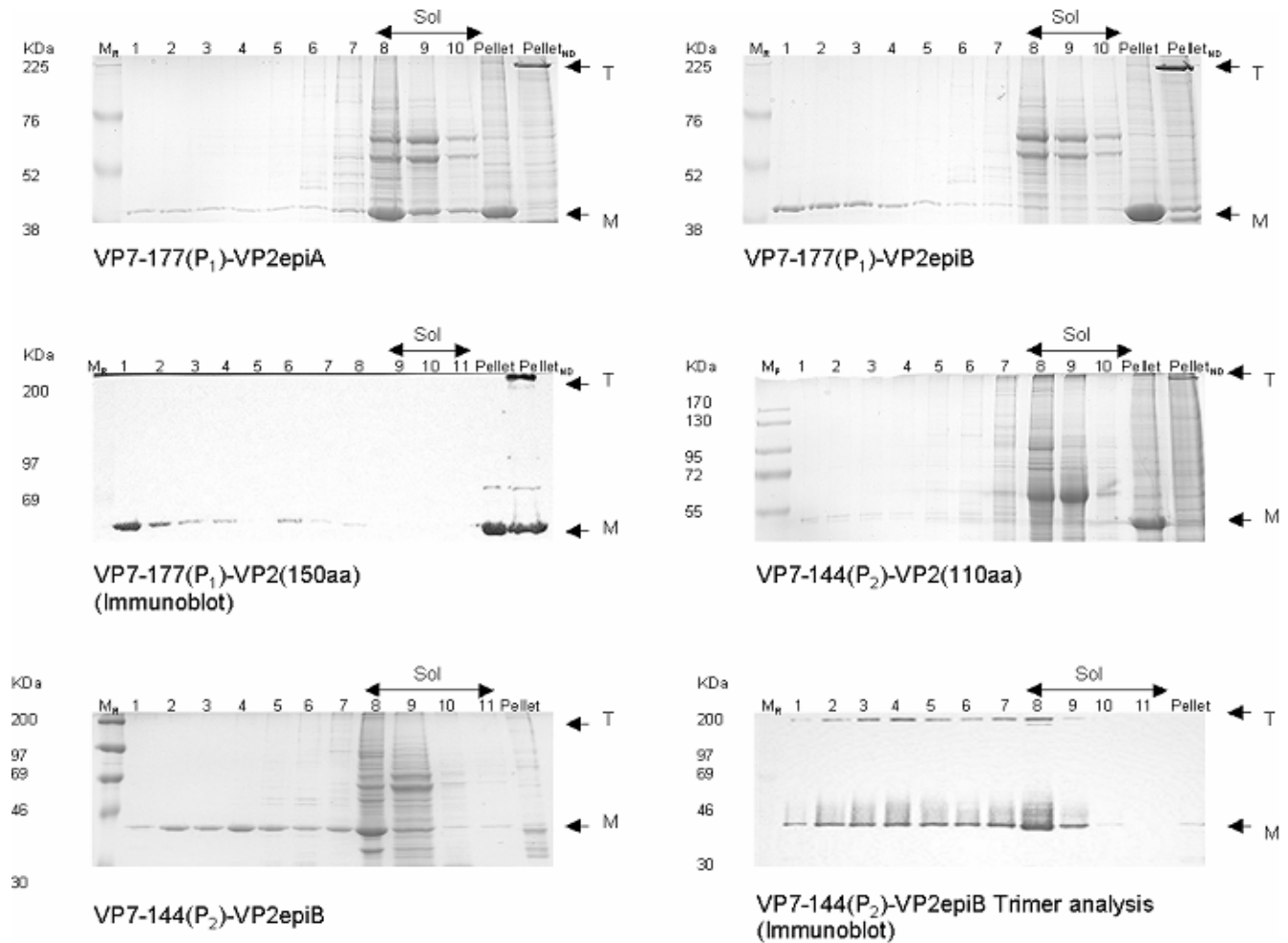


Fig 3.5: SDS-PAGE and immunoblot analyses of the sucrose gradient fractions following sedimentation analysis of cellular lysates containing proteins VP7-177(P₁)-VP2epiA (42kDa), VP7-177(P₁)-VP2epiB (42kDa), VP7-177(P₁)-VP2(150aa) (56kDa), VP7-144(P₂)-VP2(110aa) (52kDa) and VP7-144(P₂)-VP2epiB (42kDa). Trimer analysis of the pellet fraction (Pellet_{ND}) was performed by incubating the protein sample in the presence of SDS at room temperature, rather than at 95°C, prior to SDS-PAGE. Each fraction of the VP7-144(P₂)-VP2epiB sucrose gradient was analysed for the presence of trimers in this manner. The VP7 protein in the immunoblots was identified with guinea pig anti-VP7 antibodies. The VP7 trimer position is indicated by a T and the monomer VP7 protein by an M. Lanes 1-11 contain fractions 1-11 of the sucrose gradient, respectively. Double-sided arrows at the top of the gel indicate the soluble fractions of the gradient. M_R and M_P are the Rainbow™ (Amersham Pharmacia) and PageRuler™ (Fementas) pre-stained molecular size protein ladders, respectively. The relevant sizes are indicated in kDa.

Table 3.1: Relative amount of soluble VP7 fusion protein in the cellular lysate 72 hours post infection

VP7 fusion protein	Percentage soluble protein at 72 hours post-infection*
VP7-177(P ₁)-FMDVepi	40% ± 2%
VP7-144(P ₂)-FMDVepi	15% ± 1.5%
VP7-177(P ₁)-VP2epiA	53% ± 2%
VP7-177(P ₁)-VP2epiB	8% ± 0.5%
VP7-177(P ₁)-VP2(150aa)	5% ± 0.5%
VP7-144(P ₂)-VP2epiB	58% ± 2%
VP7-144(P ₂)-VP2(110aa)	8% ± 1%
VP7-144(P ₂)-ELDKWA	4% ± 0.5%
VP7-177(P ₁)-ELDKWA	19% ± 2%

*Calculated using the EZQuant software package (EZQuant Ltd)

It was also of interest to determine the effect of the AHSV VP2 peptide insertions on the ability of the VP7 protein to form trimers. Following a modified SDS-PAGE protocol, the VP7-177(P₁)-VP2epiA protein in pellet fraction of the sucrose gradient shifted from the 42KDa monomeric position to a position just below the 225KDa protein marker (Fig 3.5). As the VP7-177(P₁) trimer had also previously migrated to approximately the same position in the SDS-polyacrylamide gel (2.3.3.3), the VP7-177(P₁)-VP2epiA multimer was assumed to be in the form of a trimer. The absence of the 42KDa VP7-177(P₁)-VP2epiA monomer in the pellet fraction also indicated that the VP7-177(P₁)-VP2epiA trimer, like the VP7-177(P₁) trimer, was stable in the presence of SDS at room temperature. The VP7-177(P₁)-VP2epiB and VP7-177(P₁)-FMDVepi (2.3.5) fusion proteins also formed trimers that were stable in SDS at room temperature. Thus the insertion of the AHSV-4 VP2 epitopes 'A' and 'B', or the 36-mer FMDV VP1 peptide, into site 177(P₁) did not compromise the ability of the VP7-177(P₁) protein to form stable trimers. The insertion of the 150-mer AHSV-4 VP2 peptide into the same site, however, resulted in the detection of two prominent VP7-specific bands within the pellet fraction following the modified SDS-PAGE

protocol. This indicated that the high molecular weight VP7-177(P₁)-VP2(150aa) trimer molecules were less stable in the presence of SDS and disassociated partially into monomers (56KDa) during electrophoresis. VP7-144(P₂) trimers have previously been shown to be less stable than VP7-177(P₁) trimers in SDS at room temperature (2.3.3.3). The VP7-144(P₂)-VP2epiB and VP7-144(P₂)-VP2(110aa) fusion proteins also migrated as both a monomer and a high molecular weight trimer within their respective SDS polyacrylamide gels (Fig 3.5). Thus the 23-mer and 110-mer peptides inserted into site 144(P₂) did not interfere with the stability of the VP7-144(P₂) trimers formed. These results indicated that the insertion of foreign peptides into the top domain did not compromise the ability of the VP7 protein to form trimers. The stability of these trimers was also dependent mainly on the VP7 vector protein used, with the VP7-177(P₁) fusion trimers being more stable than the VP7-144(P₂) fusion trimers in the presence of SDS. The one exception was the VP7-177(P₁)-VP2(150aa) protein which formed trimers that were less stable than the VP7-177(P₁) vector trimer in SDS detergent, suggesting that the peptide inserted may also influence VP7 trimer stability.

The influence of the insertion site and inserted peptide on VP7 fusion protein solubility was further investigated by the insertion of various HIV-1 gp41 peptides into different sites within the VP7 top domain.

3.3.1.3 Solubility of the VP7-HIV-gp41 fusion proteins

To further investigate the effect of the site of insertion on VP7 protein solubility, the broadly neutralizing HIV-1 subtype B ELDKWA epitope (Dong et al., 2001, Muster et al., 1993) (Fig 5.1) was inserted into sites 177(P₁) and 144(P₂) (Meyer, 2002) (Fig 3.2). The effect of the peptide on VP7 fusion protein solubility was also further investigated by the insertion of the ELDKWA epitope together with the amino acids flanking this sequence within the native HIV-1 subtype B gp41 protein into site 177(P₁). Also inserted into this site was the HIV-1 subtype B RILAVEYLKD epitope (Chen & Dierich, 1998, Dong et al., 2001) together with the amino acids flanking this sequence within the native subtype B gp41 protein. The additional flanking amino acids were inserted in order for the epitope to be presented more optimally to the immune system in the context of a larger peptide. These flanking regions differed in sequence and were designated f₁-f₈ (Table 2.2). The HIV-1 subtype C sequence, equivalent to the HIV-1 subtype B GCSGKLICTT epitope (Cotropia et al., 1996, Viveros et al., 2000), was also inserted into site 177(P₁). As a greater number of surface exposed, hydrophilic amino acid residues have been shown to enhance protein solubility (Bianchi et al., 1994, Mosavi & Peng, 2003), this sequence was inserted with 6 highly hydrophilic arginine residues flanking both the N- and C-terminals to enhance VP7 protein solubility. The solubility of the resulting VP7-HIV-gp41 fusion proteins (Table 2.2) was assessed at 72 hours post-infection as described in Materials and Methods (3.2.2) under standardized experimental conditions and the resulting SDS-PAGE gels shown in Fig 3.6. The solubility of the VP7-177(P₁)-ELDKWA and VP7-144(P₂)-ELDKWA fusion proteins is indicated in Table 3.1. In some cases, the identity of the VP7 bands was established by immunoblotting with VP7-specific antibodies.

The insertion of the HIV-1 subtype B ELDKWA epitope into site 177(P₁) resulted in approximately 19% of VP7-177(P₁)-ELDKWA sedimenting in the soluble fractions. An immunoblot confirmed this result but is not shown. However, insertion of the same peptide into site 144(P₂) resulted in the VP7-144(P₂)-ELDKWA fusion protein having a solubility of only 4%. This result once again illustrated the effect of the insertion site on VP7 fusion protein solubility.

The insertion of the HIV-1 subtype B ELDKWA epitope, together with its flanking amino acids (54aa peptide), into site VP7-177(P₁) resulted in very little soluble VP7-177(P₁)-f₃ELDKWaf₄ fusion protein. This was also the case when the HIV-1 subtype B RILAVEERYLKD epitope was inserted together with its flanking amino acids (57aa peptide) into the same site. The insertion of the HIV-1 subtype C epitope-equivalent sequence, with hydrophilic arginine residues on its N- and C-terminals, into site 177(P₁) also resulted in very little VP7-177(P₁)-RCL3R fusion protein sedimenting in soluble fractions. Although this result is yet to be confirmed by means of immunoblot, this preliminary data indicated that merely increasing the hydrophilic character of the inserted peptide did not automatically result in an increase in VP7 fusion protein solubility. Alternate strategies to increase VP7 protein solubility were subsequently investigated.

3.3.2 Factors that affect VP7 solubility

The distribution of VP7-144(P₂)-FMDVepi (3.3.1.1), and other VP7 fusion proteins, throughout the length of their respective sucrose gradients may be the result of trimer-trimer aggregation. Limited VP7 fusion protein trimer-trimer aggregation may be reversed by various strategies that could include lowering the VP7 protein concentration via sample dilution and/or treatment of the VP7 aggregates with a non-ionic detergent such as Nonidet®-P40 (NP-40) or a mild ionic detergent such as sarkosyl. Other treatments, such as the amino acid L-Arginine, also need to be considered.

3.3.2.1 Treatment via protein dilution

It has been shown that, when expressed early in the infection cycle, the majority of the VP7-177(P₁)-eGFP fusion protein is soluble (Mizrachi, 2008). This is likely due to the low amount of expressed protein. As the total amount of the expressed VP7-177(P₁)-eGFP protein increases with time post infection this protein tends to aggregate into insoluble particles. It was thus investigated whether dilution of the VP7 protein solution could reverse this aggregation and, hence, increase VP7 protein solubility. Sf9 cells expressing the VP7-177(P₁)-FMDVepi fusion protein were lysed in different volumes of cell lysis buffer. More specifically, cells expressing VP7-177(P₁)-FMDVepi were harvested 72 hours post-infection, when VP7 aggregation was assumed to be at a maximum, resuspended in lysis buffer (0.01M STE, 0.5% NP-40) and aliquoted into three parts. One part was diluted 1:1 with 0.01M STE, the second part was diluted 1:2 with 0.01M STE while the third part remained

undiluted. The cells were homogenized and the VP7 protein solubility analysed as described in Materials and Methods (3.2.2). The resulting SDS-PAGE gels are shown in Fig 3.7.

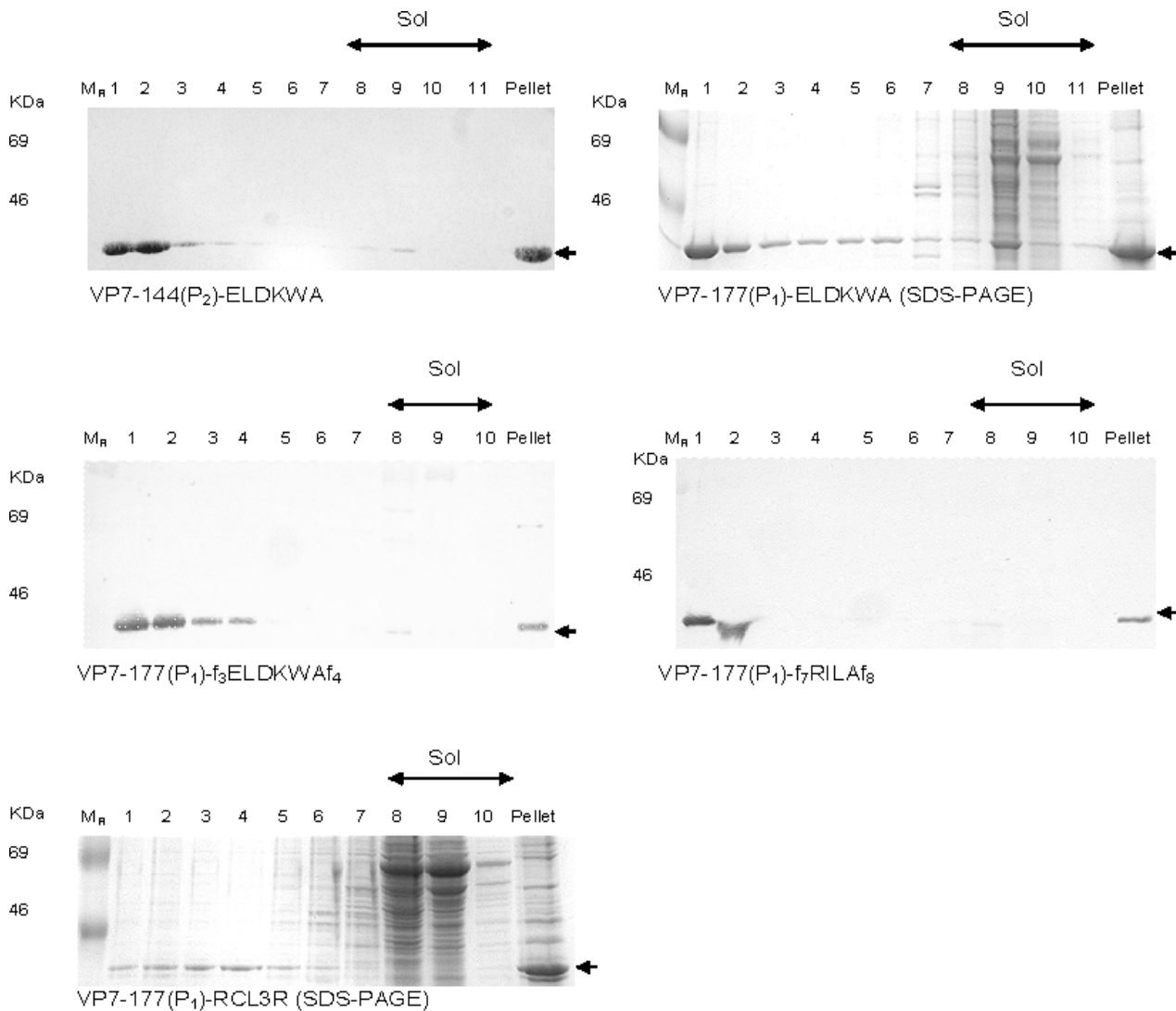


Fig 3.6: SDS-PAGE and immunoblot analysis of the sucrose gradient fractions following sedimentation analysis of cellular lysates containing proteins VP7-144(P₂)-ELDKWA (40 kDa), VP7-177(P₁)-ELDKWA (40 kDa), VP7-177(P₁)-f₃ELDKWaf₄ (45 kDa), VP7-177(P₁)-f₇RILAf₈ (44 kDa) and VP7-177(P₁)-RCL3R (41 kDa). Immunoblots were performed using guinea-pig α VP7-177 antiserum. Lanes 1-11 contain fractions 1-11 of the sucrose density gradients, respectively. Double-sided arrows at the top of the gels/membrane indicate the soluble fractions of the gradient. The arrow on the side indicates, in each case, the position of the VP7 protein on the gel/membrane. M_R is the RainbowTM coloured protein molecular weight marker (Amersham Pharmacia). The relevant sizes are indicated in kDa.

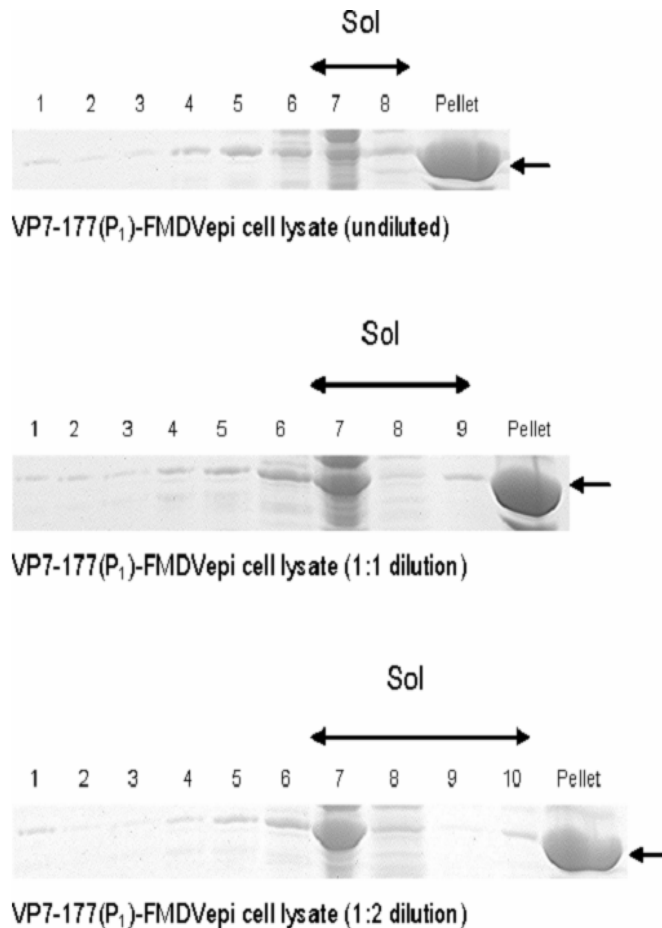


Fig 3.7: SDS-PAGE analyses of the sucrose fractions obtained following sedimentation analysis of the VP7-177(P₁)-FMDVepi-containing cellular lysate, undiluted, diluted 1:1 or diluted 1:2 with 0.01M STE. Lanes 1-10 contain fractions 1-10 of the sucrose gradient, respectively. Double-sided arrows at the top of the gel indicate the soluble fractions of the gradient while the position of the VP7 fusion protein on the gel is indicated by an arrow.

Lysis of the Sf9 cells in a small volume of cell lysis buffer (undiluted in this experiment) resulted in the bulk of the expressed VP7-177(P₁)-FMDVepi protein sedimenting to the pellet of the sucrose gradient. Only 7.5% of the protein was soluble and sedimented in sucrose fractions 7 and 8. A 1:1 dilution of the sample resulted in 24% of the VP7-177(P₁)-FMDVepi protein being soluble and sedimenting in sucrose fractions 7-9. The solubility of the VP7-177(P₁)-FMDVepi protein further increased following a 1:2 dilution of the sample with 30% of the protein sedimenting in sucrose fractions 7-10. These results indicated that diluting the VP7 protein solution decreases the VP7 concentration, effectively reducing the interactions between the protein molecules, and results in an increase in VP7 protein solubility. The discrepancy between the solubility of the VP7-177(P₁)-FMDVepi protein observed in Fig 3.4 (40%) and the solubility of this protein in the undiluted sample in Fig 3.7 (7.5%) was most likely due to the total amount of expressed VP7-177(P₁)-FMDVepi protein being much greater in the same volume of buffer in the latter experiment. This higher concentration of VP7 protein is likely to have resulted in the increased levels of protein aggregation and thus the lower VP7-177(P₁)-FMDVepi solubility observed in Fig 3.7.

Alternate strategies for reducing the interactions between the VP7 trimers, and hence reversing protein aggregation, were also investigated in this study. These included treatment with non-ionic detergent Nonidet[®]-P40 (NP-40), anionic detergent Na⁺ *N*-dodecanoylsarcosinate (Sarkosyl) and amino acid L-Arginine.

3.3.2.2 Treatment with non-ionic detergent

Nonidet[®]-P40 (NP-40) is routinely added to the cell lysis buffer in our laboratory. The effect of this non-ionic detergent on the solubility of the expressed VP7 protein was investigated. Fusion protein VP7-177(P₁)-FMDVepi was harvested 72 hours post-infection from recombinant baculovirus-infected insect cells. The cells were resuspended in either 0.01M STE buffer or STE buffer containing Nonidet[®]-P40 (0.01M STE, 0.5% NP-40). Following cell homogenization VP7 protein solubility was assessed as described in Materials and Methods (3.2.2) and the resulting SDS-PAGE gels are shown in Fig 3.8.

Lysis of the Sf9 cells in 0.01M STE buffer resulted in the bulk of the VP7-177(P₁)-FMDVepi protein being located in fractions 3-9 of the sucrose gradient. Addition of Nonidet[®]-P40 to the lysis buffer, however, resulted in the particulate VP7-177(P₁)-FMDVepi protein sedimenting mainly in fractions 1-4 and pellet of the sucrose gradient. The quantity and distribution of the soluble VP7-177(P₁)-FMDVepi protein (fractions 8-9) was not affected. Thus, the presence of the non-ionic detergent Nonidet[®]-P40 (NP-40) in the lysis buffer had no effect on the solubility of the VP7-177(P₁)-FMDVepi protein. Next it was investigated how treatment of VP7 with the solubilization agents L-arginine and Na⁺ *N*-dodecanoylsarcosinate (Sarkosyl) within the cellular lysate affected VP7 protein solubility.

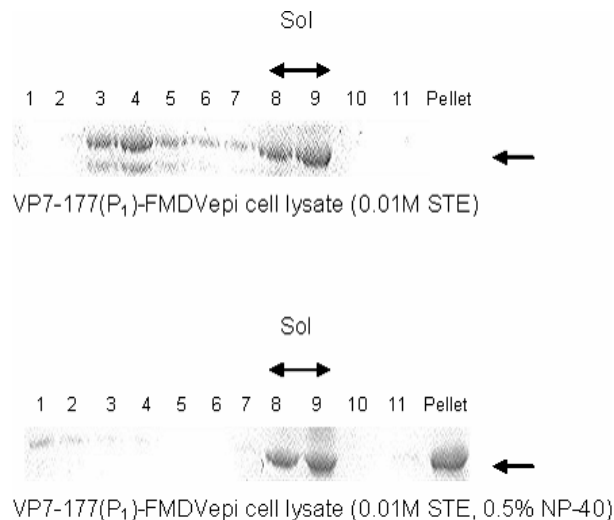


Fig 3.8: SDS-PAGE analyses of the sucrose fractions obtained following sedimentation analysis of the VP7-177(P₁)-FMDVepi-containing cellular lysate, obtained via lysis in either 0.01M STE or 0.01M STE;0.5% NP-40. Lanes 1-11 contain fractions 1-11 of the sucrose gradient, respectively. Double-sided arrows at the top of the gel indicate the soluble fractions of the gradient while the position of the VP7 fusion protein on the gel is indicated by an arrow.

3.3.2.3 Treatment of the cellular lysate with L-arginine or sarkosyl

The amino acid L-arginine (Arakawa et al., 2007, Tsumoto et al., 2004) and the ionic detergent Na⁺ *N*-dodecanoylsarcosinate (Sarkosyl) (Frankel et al., 1990, Frankel, 1991, McNally et al., 1991) are well known for their protein solubilizing ability. Although the VP7-177(P₁)-FMDVepi protein had a soluble component of approximately 40% under certain experimental conditions (3.3.1.1), a considerable quantity of this protein sedimented into the pellet of the sucrose gradient during centrifugation. To determine whether this particulate VP7-177(P₁)-FMDVepi protein could be solubilized, Sf9 insect cells containing VP7-177(P₁)-FMDVepi protein were harvested 72 hours post infection and resuspended either in lysis buffer (0.01M STE, 0.5% NP-40), lysis buffer containing 1M L-arginine or lysis buffer containing 0.5% sarkosyl. Following cell homogenization VP7-177(P₁)-FMDVepi protein solubility was assessed as described in Materials and Methods (3.2.3) and the resulting SDS-PAGE gels are shown in Fig 3.9.

Lysis of cells in the lysis buffer (0.01M STE, 0.5% NP-40) resulted in approximately 14% of the expressed VP7-177(P₁)-FMDVepi protein sedimenting in soluble sucrose fractions 8-10, with the vast majority of this protein sedimenting into the pellet of the sucrose gradient. The VP7-177(P₁)-FMDVepi protein in fractions 4-7 was hypothesized to be soluble trimers that had formed larger aggregates due to the large amount of VP7-177(P₁)-FMDVepi protein expressed within the cell. The addition of the amino acid L-arginine to the lysis buffer resulted in a significant increase in the quantity of VP7-177(P₁)-FMDVepi protein (74%) sedimenting in the soluble fractions and a corresponding decrease in the quantity of particulate VP7 protein in the pellet. This indicated that the particulate VP7-177(P₁)-FMDVepi protein in the pellet could be solubilized by the addition of L-Arginine. The addition of sarkosyl to the lysis buffer, however, resulted in the disappearance of a large amount of VP7-177(P₁)-FMDVepi protein from the sucrose gradient (result not shown). The action of proteases within the cellular lysate on sarkosyl-treated VP7-177(P₁)-FMDVepi protein was thought to be responsible for this occurrence and will be further investigated in Chapter 4. The discrepancy observed between the VP7-177(P₁)-FMDVepi solubility obtained prior to Arginine treatment (14%) and that obtained in section 3.3.1.1 (40%) was hypothesized to be due to the larger amount of VP7-177(P₁)-FMDVepi protein being expressed within the cell in this experiment, as previously discussed (3.3.2.1).

Although the addition of L-arginine to the lysis buffer solubilized VP7-177(P₁)-FMDVepi aggregates, the presence of proteases and additional cellular and viral proteins within the cellular lysate environment complicated this strategy of increasing VP7 protein solubility. Isolation of particulate VP7 aggregates from the cellular lysate prior to solubilization with L-arginine and Sarkosyl was suggested as an alternate means of obtaining large quantities of soluble VP7 fusion protein free from the bulk of cellular and viral proteins and was subsequently investigated.

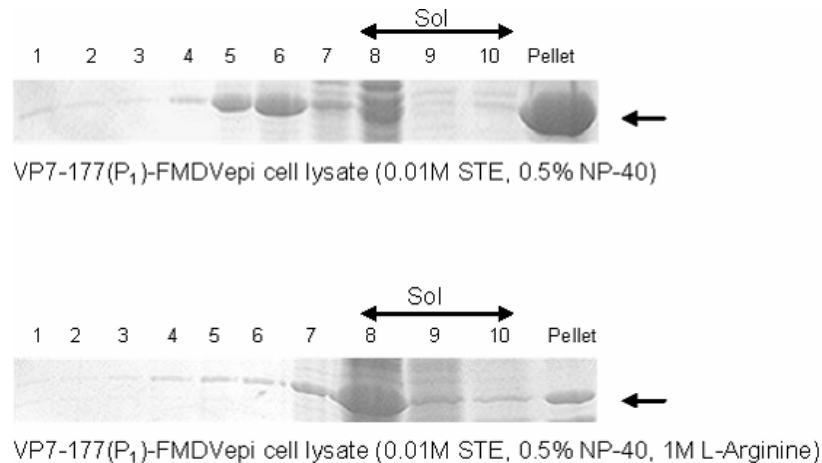


Fig 3.9: SDS-PAGE analyses of the sucrose fractions obtained following VP7-177(P₁)-FMDVepi sedimentation analysis. Cells were lysed in standard lysis buffer (0.01M STE; 0.5% NP-40) or lysis buffer to which 1M L-Arginine was added. Lanes 1-10 contain fractions 1-10 of the sucrose gradient, respectively. Double-sided arrows at the top of the gel indicate the soluble fractions of the gradient while the position of the VP7 fusion protein on the gel is indicated by an arrow.

3.3.2.4 Treatment of the particulate VP7 fraction with L-arginine or sarkosyl

The VP7-177(P₁)-FMDVepi particulate fraction was isolated as described in Materials and Methods (3.2.3) and resuspended in either 1xPBS buffer, 1xPBS buffer with 1M L-arginine or 1xPBS buffer with 0.5% Sarkosyl (Fig 3.10).

Resuspension of VP7-177(P₁)-FMDVepi aggregates in 1xPBS buffer resulted in a turbid protein suspension being observed. The addition of sarkosyl almost completely cleared the solution. L-arginine treatment also visibly reduced turbidity but less so than in the case of sarkosyl. The solubility of the untreated and treated VP7-177(P₁)-FMDVepi protein aggregates was then analysed on sucrose density gradients as described in Materials and Methods (3.2.3). The solubility of VP7-144(P₂)-FMDVepi aggregates, treated in the same manner, was also assessed and the resulting SDS-PAGE gels are shown in Fig 3.11.

The VP7-177(P₁)-FMDVepi and VP7-144(P₂)-FMDVepi aggregates were partially solubilized via resuspension in 1xPBS buffer, with approximately 40% of these VP7 fusion proteins sedimenting in soluble sucrose fractions 8-10. The bulk of the remaining protein sedimented into the pellet of these sucrose gradients. Treatment of these aggregates with 1M L-arginine resulted in the majority of the VP7-177(P₁)-FMDVepi and VP7-144(P₂)-FMDVepi protein sedimenting within the soluble sucrose fractions. Treatment with the ionic detergent sarkosyl solubilized the VP7-177(P₁)-FMDVepi and VP7-144(P₂)-FMDVepi aggregates to a greater degree with almost all the VP7 fusion protein located within the soluble fractions.

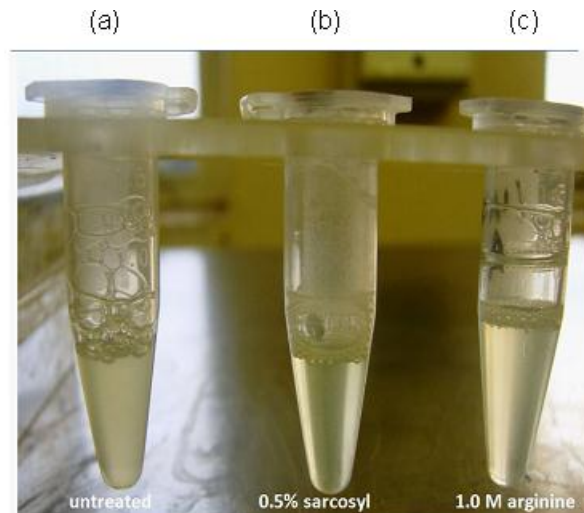


Fig 3.10: Visual effect of L-arginine and sarkosyl treatment on VP7-177(P₁)-FMDVepi particulate protein suspensions. Particulate VP7-177-FMDVepi protein was resuspended in 1xPBS buffer and left untreated (a), treated with 0.5% sarkosyl (b) or treated with 1M arginine (c).

No disappearance of the VP7-177(P₁)-FMDVepi and VP7-144(P₂)-FMDVepi proteins from the sucrose gradient fractions was observed following sarkosyl treatment of the trimer-trimer aggregates. Thus it seemed that the removal of the aggregates from the protease-containing cellular environment may have prevented this from occurring. These results indicated that VP7-177(P₁)-FMDVepi and VP7-144(P₂)-FMDVepi aggregates isolated from the cellular lysate may be successfully solubilized via treatment with the solubilization agents L-arginine and sarkosyl. This result prompted an investigation to determine whether the aggregation of other VP7 fusion proteins could be partially or completely reversed via L-arginine or sarkosyl treatment.

The particulate components of the VP7-177(P₁)-VP2epiA, VP7-177(P₁)-VP2epiB, VP7-144(P₂)-VP2(110aa) and VP7-177(P₁)-VP2-150aa fusion proteins, displaying the AHSV-4 VP2 'A' (aa321-339) and 'B' (377-400) epitopes (Fig 3.3), were harvested as described in Materials and Methods and treated with 1M L-arginine or 0.5% sarkosyl (3.2.3). Their solubility was subsequently analysed on sucrose density gradients and the resulting SDS-PAGE gels are shown in Fig 3.12.

The VP7-177(P₁)-VP2epiA, VP7-177(P₁)-VP2epiB and VP7-144(P₂)-VP2(110aa) trimer aggregates were solubilized to various degrees following 1M L-arginine treatment. The VP7-177(P₁)-VP2(150aa) trimer aggregates, although not solubilized via L-Arginine treatment, were solubilized via treatment with the ionic detergent sarkosyl. The possibility that the mild solubilization agent L-arginine solubilizes mostly correctly folded VP7 trimers while the more effective solubilization agent sarkosyl solubilizes both correctly and incorrectly folded trimers was subsequently investigated by determining whether the VP2 150aa peptide presented by the VP7-177(P₁)-VP2(150aa) fusion protein was correctly folded and immunogenic.

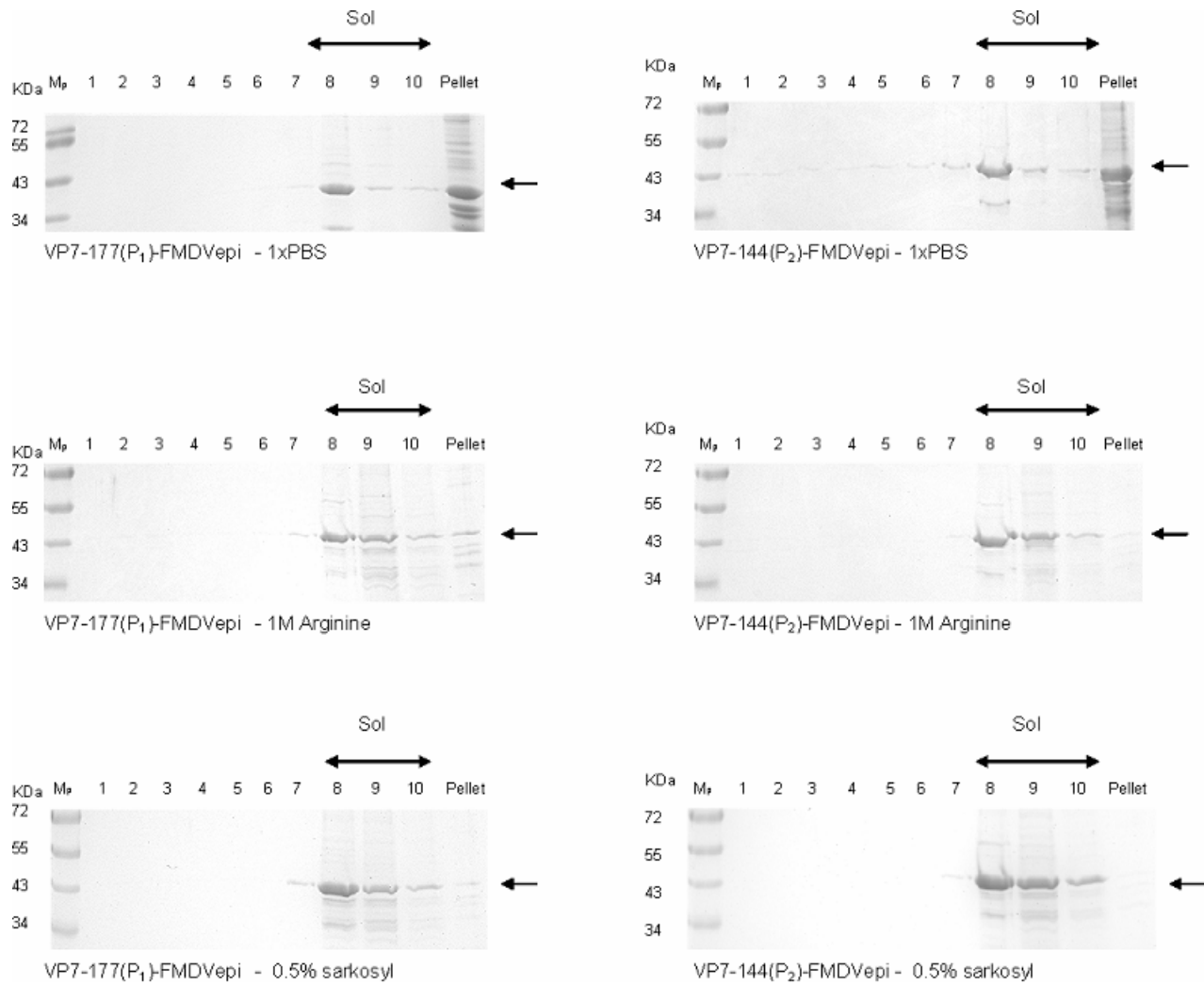


Fig 3.11: SDS-PAGE analysis of the fractions obtained following sucrose gradient sedimentation separation of the particulate VP7-177(P₁)-FMDVepi or VP7-144(P₂)-FMDVepi protein after resuspension in 1xPBS buffer, 1xPBS buffer with 1M L-arginine or 1xPBS buffer with 0.5% sarkosyl. Lanes 1-10 contain fractions 1-10 of the sucrose gradient, respectively. Double-sided arrows at the top of the gel indicate the soluble fractions of the gradient. M_p is the PageRuler™ (Fermentas) prestained molecular size protein ladder. The relevant sizes are indicated in kDa. The arrow on the side of the gel indicates the position of a unique protein corresponding to the expected size of the VP7-FMDV fusion protein.

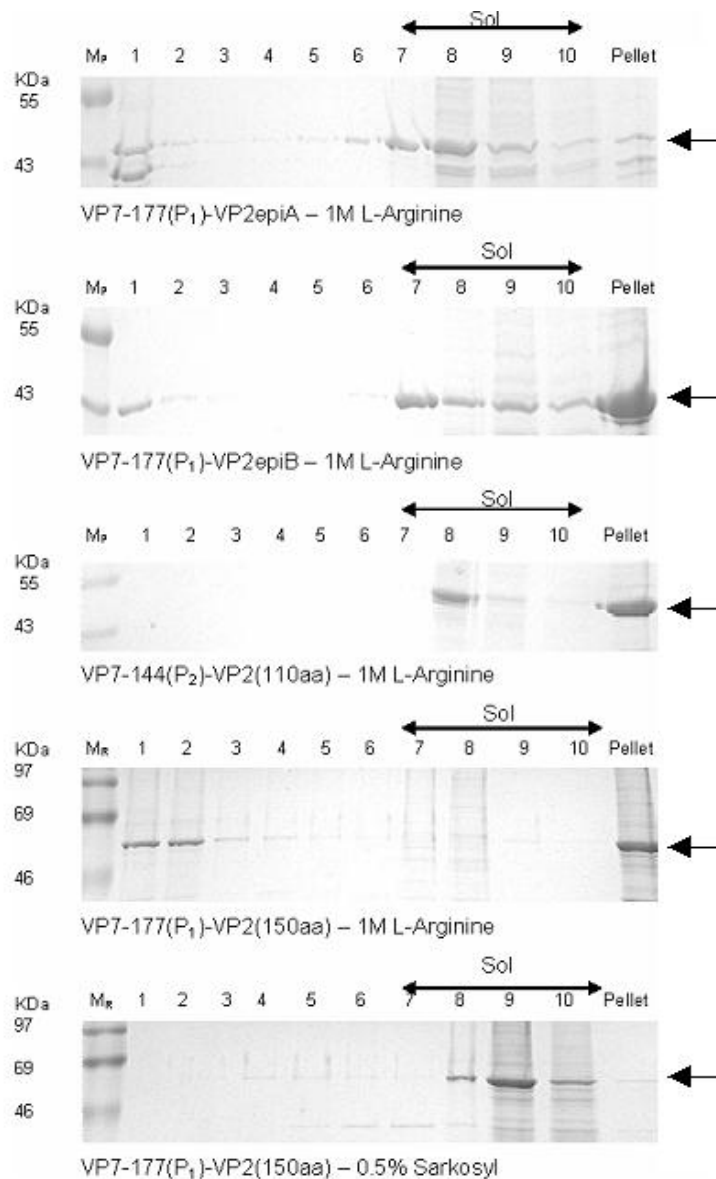


Fig 3.12: SDS-PAGE analysis of the fractions obtained following sucrose gradient sedimentation separation of the particulate VP7-177(P₁)-VP2epiA, VP7-177(P₁)-VP2epiB, VP7-144(P₂)-VP2(110aa) and VP7-177(P₁)-VP2(150aa) protein after resuspension in 1xPBS buffer with 1M L-arginine or 0.5% Sarkosyl. Lanes 1-10 contain fractions 1-10 of the sucrose gradient, respectively. Double-sided arrows at the top of the gel indicate the soluble fractions of the gradient. M_R and M_P are the Rainbow™ (Amersham Pharmacia) and PageRuler™ (Fementas) prestained molecular size protein ladders, respectively. The relevant sizes are indicated in KDa. The arrow on the side of the gel indicates the position of a unique protein corresponding to the expected size of the VP7-VP2 fusion protein.

3.3.3 Immune response against L-arginine- and sarkosyl-solubilized VP7 fusion protein

L-arginine and/or sarkosyl treatments had been shown to solubilize VP7 fusion protein trimer aggregates. However, the effect of these treatments on the immunogenicity of foreign peptides presented on the surface of the VP7 trimer was not known. Thus two animal trials were conducted in guinea pigs. The first animal trial aimed to determine whether a significant humeral immune response was elicited against L-arginine and/or sarkosyl-solubilized VP7-177(P₁)-FMDVepi trimers. The effect of antigen dose and adjuvant on the immune response was also investigated during the course of this trial. The second animal trial aimed to elucidate whether an immune response could be elicited against L-arginine- and/or sarkosyl-solubilized VP7-VP2 trimers, including the sarkosyl-solubilized VP7-177(P₁)-VP2(150aa) trimer.

3.3.3.1 Immune response against L-arginine- and sarkosyl-solubilized VP7-177(P₁)-FMDVepi fusion protein

Soluble VP7-177(P₁)-FMDVepi trimers were found capable of eliciting significant titers of insert-specific, FMDV neutralizing antibodies in an earlier guinea pig trial (2.3.7). In order to determine how this humeral immune response was affected when the VP7-177(P₁)-FMDVepi trimers were solubilized via L-arginine or sarkosyl treatment, antigen was prepared and quantified as described in section 3.2.4. Guinea pigs were inoculated with 20µg, 50µg or 80µg of antigen in the presence of either Complete Freund's adjuvant (CFA), a combination of aluminium hydroxide (Al(OH)₃) and saponin adjuvants or Montanide ISA 206B adjuvant (3.2.5). Although incomplete Freund's adjuvant had earlier in this study been found to be a satisfactory alternative to CFA (2.3.7), all previous animal trials had been performed with CFA and it was decided to apply the standard protocol. All animals received a boost 3 weeks later with the same antigen dose and adjuvant. The FMDV neutralization titers and FMDV-specific ELISA titers in the sera were determined 3 weeks later on day 42 of the animal trial (3.2.6). The neutralization titers shown in Table 3.2 reflected the ELISA titers obtained which are not shown.

The average FMDV neutralization titer elicited by 20µg L-arginine-solubilized VP7-177(P₁)-FMDVepi fusion protein, in the presence of complete Freund's adjuvant, indicated that the L-Arginine treatment did not affect the immunogenicity of the FMDV peptide and it was still able to elicit a significant humeral immune response. The average neutralization titer elicited by 20µg sarkosyl-solubilized VP7-177(P₁)-FMDVepi fusion protein, in the same adjuvant, was similar indicating that treatment with this detergent also had little effect on the immunogenicity of the FMDV peptide. This may be due to the sarkosyl concentration not being high enough to affect the conformation of non-linear, neutralizing FMDV epitopes on the surface of the VP7 trimer and/or to the presence of linear, neutralizing epitopes on the FMDV peptide that remain immunogenic even in the presence of a detergent such as sarkosyl. As both the arginine-solubilized and sarkosyl-solubilized VP7-177(P₁)-FMDVepi trimers elicited equivalent humeral immune responses, it was concluded that the mild, non-denaturing solubilizing agent L-arginine was as effective as sarkosyl detergent in solubilizing the antigen and eliciting a humeral immune response. Interestingly, the average neutralization titers elicited by 20µg of arginine and/or

sarkosyl-solubilized VP7-177(P₁)-FMDVepi protein were higher than those elicited by 40µg of untreated soluble VP7-177(P₁)-FMDVepi, with the standard deviation from these averages being smaller in the former (Table 2.4). Although the neutralization titers from the two animal trials might not be directly comparable, the smaller standard deviations obtained with the arginine and/or sarkosyl-solubilized protein indicated better reproducibility and consistency in the immune response elicited. This suggested a significant advantage in presenting soluble antigen to the immune system in the presence of agents that prevent or reverse protein aggregation, such as L-arginine or sarkosyl.

Table 3.2: Immune response in guinea pigs against arginine or sarkosyl solubilized VP7-177(P₁)-FMDVepi antigen in the presence of different adjuvants

Antigen	Antigen Dose	Adjuvant	FMDV Neutralization Titres: Log ₁₀ reciprocal	
			42 days p.i	Average ^(a)
VP7-177(P ₁)- FMDVepi (Arginine Solubilized)	20µg	Complete Freunds	3.2 3.4 3.0 2.9 2.7	3.02 (±0.11)
	20µg	Combination AIOH and Saponin	1.3 1.6 1.1 2.3	1.6 (±0.53)
	50µg	Combination AIOH and Saponin	2.4 1.8 2.4 2.1	2.2 (±0.29)
	80µg	Combination AIOH and Saponin	2.4 2.8 2.0 2.4	2.4 (±0.35)
	20µg	Montanide ISA 206B	3.0 2.3 3.1 3.1	2.9 (±0.39)
	80µg	Montanide ISA 206B	3.0 2.5 3.1 3.2	3.0 (±0.31)
VP7-177(P ₁)- FMDVepi (Sarkosyl Solubilized)	20µg	Complete Freunds	2.91 3.11 3.09 3.16 2.86	3.04 (±0.27)

^(a) Standard deviation of averages at 42 days p.i in brackets

The FMDV neutralizing antibody titer elicited by 20µg of arginine-solubilized VP7-177(P₁)-FMDVepi in the presence of Montanide ISA 206B adjuvant was very similar to the titer elicited by 20µg of arginine-solubilized antigen in complete Freund's adjuvant. This indicated that the Montanide ISA 206B adjuvant was as effective at enhancing the immune response against the antigen as the Complete Freund's adjuvant, known to cause severe adverse localized reactions in animals. Twenty micrograms of arginine-solubilized VP7-177(P₁)-FMDVepi, in the presence of Al(OH)₃/Saponin combination adjuvant, however, elicited a much lower FMDV neutralizing antibody titer. Although this titer increased as the antigen dose increased, the titer elicited against 80µg arginine-solubilized VP7-177(P₁)-FMDVepi in the Al(OH)₃/Saponin combination adjuvant was still lower than that elicited against 20µg of the antigen in Montanide ISA 206B. The decreased ability of the Al(OH)₃/Saponin combination adjuvant to enhance the immune response against the antigen precluded its use in any further animal trials. No significant increase in the neutralization titer was observed following an increase in the arginine-solubilized VP7-177(P₁)-FMDVepi dose when this antigen was inoculated in the presence of Montanide ISA 206B adjuvant. Thus only 20µg arginine-solubilized VP7-177(P₁)-FMDVepi was required to elicit an immune response equivalent to that elicited by 80µg of this antigen when administered in the presence of Montanide ISA 206B adjuvant.

3.3.3.2 Immune response against L-arginine and sarkosyl-solubilized VP7-VP2 fusion protein

As L-arginine and sarkosyl treatments did not affect the immunogenicity of the FMDV peptide presented on the surface of the VP7 trimer (3.3.3.1), it was investigated whether the 'A' (aa321-339) and 'B' (377-400) epitopes of AHSV-4 VP2 were also immunogenic when presented on the surface of L-arginine- or sarkosyl-solubilized VP7 trimers. These epitopes were presented individually (VP7-177(P₁)-VP2epiA; VP7-177(P₁)-VP2epiB) or in combination with each other on larger VP2 peptides (VP7-144(P₂)-VP2(110aa); VP7-177(P₁)-VP2(150aa) (Fig 3.3). The VP7-VP2 fusion proteins were prepared and quantified as described in section 3.2.4. Guinea pigs were inoculated with 40µg of these antigens in the presence of Complete Freund's adjuvant (3.2.5). Although 20µg of L-arginine- or sarkosyl-solubilized antigen in Complete Freund's adjuvant has been found to elicit an adequate humoral immune response (3.3.3.1), the immunogenicity of the VP7-VP2 fusion proteins was not known and, in order to eliminate the possibility of insufficient antigen, a higher antigen dose (40µg) was used in this animal trial. All animals received a boost 3 weeks later with the same antigen dose and adjuvant. The VP2-specific ELISA titers and the AHSV neutralization titers in the sera were determined 3 weeks later on day 42 of the animal trial (3.2.6) and are shown in Table 3.3.

The arginine-solubilized VP7-177(P₁)-VP2epiA fusion protein did not elicit significant titers of AHSV-4 VP2 epitope 'A'-specific antibodies, as measured by a VP2epiA -specific ELISA assay. As the inserted AHSV VP2 'A' peptide (aa321-339) has previously been reported to contain the neutralizing 'A' linear epitope (Martinez-Torrecuadrada et al., 2001), it is unclear why this peptide failed to induce an insert-specific humoral immune response. The arginine-solubilized VP7-177(P₁)-VP2epiB and VP7-144(P₂)-VP2(110aa) proteins, however, elicited AHSV-4 VP2 epitope 'B'-specific antibodies, with individual titers as high as 4.5, when measured by a

VP2epiB-specific ELISA. Thus the linear AHSV VP2 'B' neutralizing epitope (Martinez-Torrecuadrada et al., 2001), when presented on its own on the surface of VP7-177(P₁)-VP2epiB trimers or in the context of the larger 110aa AHSV-4 VP2 peptide on the surface of VP7-144(P₂)-VP2(110aa) trimers, was immunogenic following L-arginine treatment. Although solubilized via sarkosyl treatment, the VP7-177(P₁)-VP2(150aa) trimers did not elicit significant titers of AHSV-4 VP2 epitope 'B'-specific antibodies as measured by a VP2epiB -specific ELISA assay. This indicated that although the sarkosyl detergent solubilized the VP7-177(P₁)-VP2(150aa) trimer-trimer aggregates (3.3.2.4), the solubilized trimers presented a misfolded 150aa VP2 peptide to the immune system. As treatment with low concentrations of sarkosyl do not affect the native form and function of a protein (Frangioni & Neel, 1993, Frankel et al., 1990, Tan & Spudich, 1991), it is likely that the linear AHSV-4 VP2 'B' epitope may have been obscured from the immune system by the incorrect folding of the 150aa VP2 peptide within the VP7 top domain. As L-arginine was not able to solubilize the VP7-177(P₁)-VP2(150aa) trimer aggregates (3.3.2.4), it is likely that this mild solubilization agent L-arginine solubilizes correctly folded VP7 trimers while the more effective solubilization agent sarkosyl solubilizes both correctly and incorrectly folded VP7 trimers.

Although AHSV-4 neutralization assays indicated that some of the guinea pigs, inoculated with the arginine-solubilized VP7-177(P₁)-VP2epiB and VP7-144(P₂)-VP2(110aa) proteins, showed titers of AHSV-4 neutralizing antibodies over and above the background titer of between 0.6 and 0.9, the titers were generally too low and variable to make a significant conclusion.

Table 3.3: VP2 insert-specific ELISA and AHSV-4 neutralizing antibody titres following inoculation of guinea pigs with L-arginine/sarkosyl-solubilized VP7-VP2 fusion proteins

Antigen	Adjuvant And Dose	VP2 insert-specific ELISA		AHSV Neutralization	
		Titres: Log ₁₀ reciprocal		Titres: Log ₁₀ reciprocal	
		42 days p.i	Average ^(a)	42 days p.i.	Average ^(a)
VP7-177(P ₁)-VP2epiA (Arginine-solubilized)	Complete Freund's (40µg)	2.7 2.7 2.7 2.7 2.7	2.7 (±0.0)	0.6 (sera pooled)	0.6 (±0.0)
VP7-177(P ₁)-VP2epiB (Arginine-solubilized)	Complete Freund's (40µg)	4.51 4.51 4.20 3.30 4.20	4.14 (±0.50)	1.20 0.90 1.20 0.90 1.20	1.08 (±0.16)
VP7-144(P ₂)-VP2(110aa) (Arginine-solubilized)	Complete Freund's (40µg)	4.20 3.30 4.20 3.00 3.00	3.66 (±0.49)	1.20 0.90 1.20 0.60 0.90	0.96 (±0.25)
VP7-177(P ₁)-VP2(150aa) (sarkosyl-solubilized)	Complete Freund's (40µg)	2.7 2.7 2.7 2.7 2.7	2.7 (±0.0)	0.6 (sera pooled)	0.6 (±0.0)

^(a) Standard deviation of the average titres at 42 days p.i in brackets

3.4 Discussion

The success of AHSV VP7 as an antigen display system depends on its ability to form soluble, stable trimers that present the inserted foreign peptide in its native immunogenic conformation to the immune system. The two factors that predominantly influence the solubility and stability of these VP7 trimers are the peptide insert and VP7 top domain insertion site (Chapter 2). In order to further investigate the effect of these factors, a range of foreign peptides were inserted into different sites within the VP7 top domain. These peptides originated from the FMDV VP1 and AHSV VP2 capsid proteins as well as the envelope protein gp41 of HIV-1 and contained previously described immunogenic epitopes or epitope-equivalent sequences.

Although 6-mer peptides inserted into the AHSV VP7 top domain did not affect the ability of this protein to form trimers, the stability of certain VP7 vector trimers in the presence of sodium dodecyl sulphate (SDS) was reduced (Chapter 2). The VP7-200(P₁) vector trimers were found to be less stable than the VP7-177(P₁) trimers in the presence of this harsh detergent indicating the influence of the insertion site on VP7 trimer stability. In this study we found that the insertion of large peptides, up to 150 amino acids in length, into the VP7 top domain (aa121-249) did not affect VP7 trimer formation. The VP7-177(P₁)-VP2epiA, VP7-177(P₁)-VP2epiB, VP7-177(P₁)-FMDVepi, VP7-177(P₁)-VP2(150aa), VP7-144(P₂)-VP2epiB and VP7-144(P₂)-VP2(110aa) fusion proteins all formed trimers. This was not surprising as trimer formation is governed mainly by the N- and C-terminals within the bottom domain of the VP7 protein (Hosamani et al., 2010, Monastyrskaya et al., 1997, Wang et al., 1994). The stability of these trimers in SDS was, however, affected in some cases and seemed to depend primarily on the insertion site. The insertion of the neutralizing AHSV-4 VP2 'A' (aa321-339) and/or 'B' (aa377-400) epitopes (Martinez-Torrecuadrada et al., 2001) or the FMDV VP1 peptide (aa129-164) (Aggarwal & Barnett, 2002, Bittle et al., 1982, Morgan & Moore, 1990, Pfaff et al., 1982, Robert, 2003, Shao et al., 2011, Zamorano et al., 1998) into site 177(P₁) (Smalle, 2006) (Dr Fick) yielded trimers that were stable in the presence of SDS. The insertion of the FMDV VP1 peptide (aa129-164), AHSV-9 VP2 'B' epitope (377-400) (Venter et al., 2000), and/or the AHSV-4 VP2(110aa) peptide, containing both the 'A' and 'B' epitopes, into site 144(P₂) resulted in the formation of trimers that were less stable. The stability of the VP7 fusion protein trimers generally reflected the stability of the VP7 vector protein trimers in SDS and emphasized the importance of the insertion site in VP7 trimer stability. Although the inserted peptide did not generally affect VP7 trimer stability, the VP7-177(P₁)-VP2(150aa) trimers were found to be less stable than the VP7-177(P₁) vector trimers in SDS. This may have been due to the large size of the inserted VP2 peptide. The effect of top domain peptide insertions on VP7 trimer stability is not completely surprising as the VP7 top domain is also thought to play a role in trimer formation (Grimes et al., 1995, Grimes et al., 1998).

The insertion of the FMDV VP1 peptide (aa129-164) into site 177(P₁) yielded the VP7-177(P₁)-FMDVepi fusion protein with a solubility of approximately 40% (Chapter 2). Here the insertion of the same 36 amino acid peptide into a different site within the VP7 top domain, site 144(P₂) (Bolton, unpublished data), resulted in the VP7-

144(P₂)-FMDVepi fusion protein having a solubility of only 15%. This result confirms the role of the VP7 top domain insertion site in VP7 fusion protein solubility. It must also be added that the flanking P₁ and P₂ peptides, inserted during VP7-177(P₁) and VP7-144(P₂) vector construction, may also play a role. A similar result was obtained following the insertion of neutralizing HIV-1 subtype B epitope ELDKWA (Dong et al., 2001, Muster et al., 1993) into sites 177(P₁) and 144(P₂) (Meyer, 2002), with the VP7-177(P₁)-ELDKWA protein having a solubility of 19% and the VP7-144(P₂)-ELDKWA protein having a solubility only 4%. The role of the inserted peptide in VP7 fusion protein solubility was demonstrated by the insertion of the FMDV peptide and/or ELDKWA peptide into site 177(P₁). The resulting VP7-177(P₁)-FMDVepi fusion protein had a solubility of 40% while the VP7-177(P₁)-ELDKWA fusion protein with a solubility of only 19%. Similarly, the VP7-144(P₂)-FMDVepi (15%) and VP7-144(P₂)-ELDKWA (4%) fusion proteins differed in solubility.

The influence of the inserted peptide on VP7 fusion protein solubility was further demonstrated by solubility assays of the VP7-VP2 fusion proteins. The insertion of the neutralizing AHSV-4 VP2 'A' epitope (aa321-339) (Martinez-Torrecuadrada et al., 2001) into site 177(P₁) (Smalle, 2006) resulted in 53% of the VP7-177(P₁)-VP2epiA protein being soluble. However, insertion of the neutralizing AHSV-4 VP2 'B' epitope (aa377-400) (Martinez-Torrecuadrada et al., 2001) into the same site (Smalle, 2006) resulted in a VP7-177(P₁)-VP2epiB protein solubility of only 8%. The VP7-177(P₁)-VP2(150aa) fusion protein, presenting a peptide overlapping both the AHSV-4 VP2 'A' and 'B' epitopes inserted into site 177(P₁), was also relatively insoluble (5%). The VP7-144(P₂)-VP2epiB (Riley, 2003, Venter et al., 2000) and VP7-144(P₂)-VP2(110aa) (Martinez-Torrecuadrada et al., 2001) fusion proteins had vastly different protein solubility, 58% and 8%, respectively. These results all support the hypothesis that the inserted peptide plays a role in VP7 fusion protein solubility.

In an attempt to increase the immunogenicity of small epitopes, the regions flanking an epitope in its respective native protein were added to the N and C terminals of the epitope sequence presented by the VP7 trimer. It was hypothesized that the display of epitopes on such scaffolds would also allow for more optimal peptide folding, the display of a greater number of hydrophilic domains to the aqueous environment and, consequently, an increase in VP7 trimer solubility. However, the insertion of the AHSV-4 VP2 'A' and 'B' epitopes into top domain of VP7 in the context of the larger AHSV-4 VP2 110aa and 150aa peptides did not yield more soluble VP7 fusion proteins. Similarly, the insertion of the HIV-1 subtype B epitopes ELDKWA (Dong et al., 2001, Muster et al., 1993) and RILAVEYLKD (Chen & Dierich, 1998, Dong et al., 2001, Shafferman et al., 1991), with their respective flanking amino acids sequences, did not result in increased VP7 fusion protein solubility. It is likely that the HIV-1 flanking sequences, forming secondary α -helices in the native gp41 ectodomain (Fig 3.14), may not be able to fold correctly when inserted into the VP7 top domain. Another strategy to increase VP7 fusion protein solubility was the insertion of highly hydrophilic arginine residues together with the epitope sequence. The mutation of surface exposed hydrophobic amino acids to polar residues had previously been shown to increase protein solubility (Bianchi et al., 1994, Mosavi & Peng, 2003). Thus the HIV-1 subtype C sequence, equivalent to the HIV-1 subtype B GCSGKLICTT epitope (Cotropia et al., 1996, Kliks et al., 1994, Viveros et al.,

2000), was inserted into site 177(P₁) with 6 highly hydrophilic arginine residues on its N-and C-terminals. The resulting VP7-177(P₁)-RCL3R protein was, however, relatively insoluble indicating that simply increasing the hydrophilic character of the inserted peptide does not ensure the presentation of hydrophilic domains to the aqueous environment and a resulting increase in VP7 fusion protein solubility.



Fig 3.14: Computer generated model of the secondary structure of the HIV-1 gp41 ectodomain (aa541-663), modelled on the secondary structure of SIV gp41 (Courtesy of Dr Meyer). Indicated are the α -helical secondary structures (red), the ELDKWA and RVLAVERYLKD epitopes (green) and amino acids without a specific structure (white).

At this stage VP7 fusion protein solubility cannot be accurately predicted and each VP7 fusion construct needs to be individually assessed. It is clear that the inserted peptide and the insertion site within the VP7 top domain each play a role, but protein solubility may also be influenced by a range of other factors including the set of experimental conditions used. An earlier study indicated that the VP7-177(P₁)-eGFP fusion protein expressed in a baculovirus-infected insect cell early in the infection cycle is predominantly soluble but tends to aggregate as the total amount of the expressed VP7 protein increases with time post infection (Mizrachi, 2008). VP7 fusion protein solubility thus changes relative to the amount of VP7 protein that is present within a cell at a particular time.

It is currently unclear what characteristics the inserted peptide must have, or into which site it should be inserted, in order to ensure VP7 fusion protein solubility. A number of VP7 fusion proteins in this study formed a range of various sized aggregates that were distributed throughout their respective sucrose gradients under the given experimental conditions. Various treatments have been suggested to reverse this VP7 protein aggregation and,

thus, increase VP7 protein solubility. These included lowering the VP7 protein concentration by means of sample dilution and treatment of VP7 aggregates with various chemicals, including the non-ionic detergent Nonidet[®] P-40 (NP-40), the anionic detergent Na⁺*N*-dodecanoylsarcosinate (Sarkosyl) and the amino acid L-Arginine. As the AHSV VP7 trimer is a relatively stable molecule, even in the presence of the harsh detergent sodium dodecyl sulphate (SDS), less harsh treatments, such as NP-40, sarkosyl and L-arginine, would most likely not affect trimer stability. The effect of these chemical treatments on the immunogenicity of the inserted peptide was, however, not known and was investigated in this study.

Dilution of the VP7-177(P₁)-FMDVepi fusion protein solution increased the yield of soluble VP7-177(P₁)-FMDVepi trimers. It is likely that the decrease in VP7 protein concentration resulted in a reduction in the VP7 trimer-trimer interactions and thus increased trimer solubility. This confirmed the results of an earlier study which indicated that VP7 trimers are more prone to aggregate when the amount of VP7 protein being expressed within the cell is high (Mizrachi, 2008). The effect of VP7 protein concentration on VP7-177(P₁)-FMDVepi fusion protein solubility was clearly evident during several experiments in this study.

The L-arginine has been shown to reduce the aggregation of a variety of proteins (Golovanov et al., 2004, Maity et al., 2009, Shubenok et al., 2009, Tsumoto et al., 2010, Tsumoto et al., 2003, Umetsu et al., 2005). The addition of L-arginine to a cellular lysate containing VP7-177(P₁)-FMDVepi protein aggregates resulted in a significant increase in the amount of soluble VP7-177(P₁)-FMDVepi trimers. The corresponding decrease in particulate VP7-177(P₁)-FMDVepi protein sedimenting into the pellet of the sucrose gradient indicated that the interactions between the aggregated VP7 trimer molecules were reduced following the addition of this solubilizing agent. Recent studies have shown that arginine's guanidinium group interacts hydrophobically with hydrophobic surfaces or aromatic residues effectively shielding these regions and preventing protein-protein aggregative interactions, resulting in an increase in protein solubility (Ito et al., 2011, Li et al., 2010).

Na⁺*N*-dodecanoylsarcosinate (Sarkosyl), a sodium salt of the alkyl anionic detergent *N*-laurylsarcosine, has also been employed in a wide spectrum of studies to solubilize protein aggregates (Burgess, 1996, Grathwohl et al., 1996, Skoglund et al., 2008). Early studies indicated that proteins solubilized by this detergent retained their native conformation and function (Frangioni & Neel, 1993, Frankel et al., 1990, Tan & Spudich, 1991). The non-denaturing effect of low sarkosyl concentrations on proteins is likely due to this detergents carboxyl end being less strongly ionized than that of the more harsh SDS detergent. Sarkosyl is thought to bind to partially folded forms of protein thus disrupting the hydrophobic interactions between them and, consequently, inhibiting their aggregation (Mitraki, 2010). In this study, the addition of sarkosyl detergent to the cellular lysate containing the VP7-177(P₁)-FMDVepi fusion protein resulted in a reduction in the amount of VP7 protein observed on the SDS-PAGE gel. It is hypothesized that proteases, present within the cellular lysate, have a greater opportunity to introduce nicks within the VP7 trimer molecule when it is solubilized by the sarkosyl detergent and this results in

VP7 protein breakdown under denaturing conditions. This effect will be discussed in more detail in Chapter 4. In contrast, the non-ionic detergent Nonidet[®] P-40 had no effect on VP7 protein solubility.

In order to prevent the introduction of site-specific nicks within the VP7 trimer molecule by cellular proteases, it was suggested that VP7 protein aggregates be isolated from the cellular lysate prior treatment with sarkosyl or L-arginine. This low speed centrifugation strategy not only ensured the removal of the cellular proteases but also the bulk of the soluble cellular and viral proteins present in the protein sample. VP7-177(P₁)-FMDVepi and VP7-144(P₂)-FMDVepi trimer aggregates were both successfully solubilized with L-arginine and/or sarkosyl treatment, with sarkosyl solubilizing the aggregates to a greater degree. The particulate component of the VP7-177(P₁)-VP2epiA, VP7-177(P₁)-VP2epiB, VP7-144(P₂)-VP2(110aa) and VP7-177(P₁)-VP2(150aa) proteins were also treated with L-arginine and/or sarkosyl and yielded varying results. Although the bulk of the VP7-177(P₁)-VP2epiA aggregates were solubilized via L-arginine treatment, the VP7-177(P₁)-VP2epiB and VP7-144(P₂)-VP2(110aa) aggregates were only partially solubilized. The VP7-177(P₁)-VP2(150aa) aggregates were not solubilized by L-arginine treatment. They were, however, solubilized by sarkosyl detergent. It is hypothesized that the VP7 fusion protein exists in three forms, soluble, correctly folded trimers, correctly folded trimers that have aggregated and misfolded or incorrectly folded trimers that have also aggregated. Sample dilution or treatment with L-arginine, a mild solubilization agent, may solubilize only loose aggregates of correctly folded trimers while the harsher sarkosyl detergent solubilizes both correctly folded trimer aggregates and misfolded trimer aggregates. To determine whether this was indeed the case, it was necessary to determine whether the arginine and/sarkosyl treated VP7-VP2 trimers were capable of eliciting a VP2-insert specific humeral immune response. This would indicate whether the VP2 peptides were correctly folded when presented on the surface of arginine and/sarkosyl solubilized VP7 trimers. It was, however, first necessary to determine whether the AHSV-4 VP2 peptides were immunogenic when presented by untreated, soluble VP7 trimers.

The effect of L-arginine and/or sarkosyl treatment on the immunogenicity of the FMDV VP1 antigenic peptide, previously found to elicit a neutralizing, humeral immune response when presented on the surface of untreated, soluble VP7 trimers (Chapter 2) was investigated. The FMDV-specific humeral immune responses elicited by L-Arginine and sarkosyl-solubilized VP7-177(P₁)-FMDVepi trimers in guinea pigs indicated that these treatments did not interfere with the immunogenicity of the FMDV VP1 peptide. The sarkosyl-solubilized and L-arginine-solubilized trimers were found to induce equivalent neutralizing, humeral immune responses. Although treatment of the VP7-177(P₁)-FMDVepi aggregates with a low concentration of sarkosyl detergent did not result in protein denaturation or affect the immunogenicity of the FMDV VP1 peptide, this detergent was required to be removed from the protein sample prior to animal inoculation. The use of L-arginine as a solubilization agent may thus be preferable as its removal is not necessary prior to inoculation. The soluble antigen may also be freeze-dried in the presence of L-Arginine and remains fully soluble following reconstitution in water. It was interesting to note that the average neutralization titers elicited by 20µg of L-Arginine and/or sarkosyl-solubilized VP7-177(P₁)-FMDVepi trimers were higher than the titers elicited by 40µg of soluble, untreated VP7-177(P₁)-FMDVepi trimers

in the previous guinea pig trial (Chapter 2). Although the titers from the two animal trials cannot be directly compared, smaller standard deviations were obtained with the arginine and/or sarkosyl-solubilized antigen. This indicated that the presentation of VP7 trimers to the immune system in combination with reagents that prevent or reverse trimer aggregation ensures trimer solubility and thus a reproducible and consistently high immune response. Although the 3 log₁₀ FMDV neutralization titers elicited by the L-Arginine and/or sarkosyl-solubilized VP7-177(P₁)-FMDVepi trimers in guinea pigs were much higher than the 2 log₁₀ neutralization titers that are normally considered sufficient to protect cattle against FMDV infection (Barnett et al., 2003), we cannot extrapolate the guinea pig results to larger animals (Alves et al., 2009, Kamstrup et al., 2006). Studies in cattle are currently being conducted.

Since the L-arginine and sarkosyl solubilizing treatments did not affect the immunogenicity of the FMDV peptide, it was assumed that these treatments would, similarly, not affect immunogenicity of the AHSV-4 VP2 peptides. Although presenting AHSV-4 VP2 neutralizing epitope 'A' (aa321-339) (Martinez-Torrecuadrada et al., 2001), the arginine-solubilized VP7-177(P₁)-VP2epiA trimers did not elicit significant titers of 'A' epitope-specific antibodies. It is not clear why this linear epitope was not immunogenic when presented on the surface of the VP7 trimer. However, the VP2 neutralizing epitope 'B' epitope (aa377-400) (Martinez-Torrecuadrada et al., 2001), presented alone or in context of the larger 110aa AHSV-4 VP2 peptide by arginine-solubilized VP7-177(P₁)-VP2epiB or VP7-144(P₂)-VP2(110aa) trimers, respectively, elicited significant titers of 'B' epitope-specific antibodies. The insolubility, and hence lack of immunogenicity, of the full length AHSV outer capsid protein VP2 and smaller AHSV VP2 antigenic domains expressed in bacterial and insect cells (Martinez-Torrecuadrada & Casal, 1995, Scanlen et al., 2002, Venter et al., 2000) have until now been a stumbling block in the search for a recombinant AHSV vaccine. The ability of VP7 trimers to display the AHSV VP2 'B' epitope in a soluble, immunogenic form to the immune system is a step closer in the development of such a vaccine. It was interesting to observe that, when presented as part of the AHSV-4 VP2 150aa peptide on the surface of sarkosyl-solubilized VP7-177(P₁)-VP2(150aa) trimers, this epitope was not able to elicit significant titers of VP2 'B'-specific antibodies. It is likely that the VP2 150aa peptide was misfolded, obscuring the linear AHSV-4 VP2 'B' epitope from the immune system, and remained misfolded even after solubilization of the VP7-177(P₁)-VP2(150aa) trimer-trimer aggregates by sarkosyl detergent.

The above results support our earlier hypothesis that the loose aggregation of correctly folded trimers, such as the VP7-177(P₁)-FMDVepi, VP7-177(P₁)-VP2epiB and VP7-144(P₂)-VP2(110aa) trimers, may be reversed by sample dilution or via treatment with a mild solubilization agent such as L-Arginine. The harsher detergent sarkosyl is, however, more effective, reversing the aggregation of both correctly folded (VP7-177(P₁)-FMDVepi) and incorrectly folded (VP7-177(P₁)-VP2(150aa)) trimer aggregates. It is, however, important to note that although sarkosyl detergent may solubilize VP7 trimer aggregates this does not necessarily mean that the inserted peptide is correctly folded and immunogenic, as in the case of the VP2(150aa) peptide, and removal of this detergent from the sample may result in re-aggregation of the incorrectly folded trimers.

Although the protein solubility studies conducted thus far give a rough estimate, true VP7 protein solubility can only be determined if the amount of correctly folded trimers, both in a soluble as well as in an aggregated form, is compared to the amount of incorrectly, folded aggregated trimers. As L-arginine only reverses the aggregation of correctly folded VP7 trimer molecules, treatment of the cellular lysate with this solubilizing agent would allow for a more accurate estimate of amount of correctly folded VP7 trimers and, thus, VP7 protein solubility.

The small animal trial conducted with L-Arginine and sarkosyl-solubilized VP7-177(P₁)-FMDVepi trimers also aimed to elucidate the effect of adjuvant and antigen dose on the immune response elicited in guinea pigs. Unlike live-attenuated and inactivated whole pathogen vaccines, most protein- and DNA-based vaccines struggle to elicit strong adaptive immune responses and long term protection (Mortellaro & Ricciardi-Castagnoli, 2011). These vaccines are normally administered in conjugation with a pharmacological or immunological agent called an adjuvant in order to enhance the immune response of the host to the inoculated antigen (Higgins & Mills, 2010). An adjuvant usually has few, if any, antigenic effects when administered on its own. Many adjuvants are thought to exert their immunostimulatory effect by promoting uptake and presentation of the processed antigen by dendritic cells followed stimulation of T-cell activation (Mosca et al., 2008, Sharp et al., 2009). Modern adjuvants belong to two main groups, the vehicles and the immunostimulants (Brunner et al., 2010). Vehicles are substances that enable optimum presentation of the vaccine antigen to the immune system and include emulsions, liposomes, virosomes, polymeric microspheres and mineral salts (aluminium or calcium phosphate). Immunostimulants enhance the immune response to the antigen directly and are often microbial products such as TLR ligands or bacterial endotoxins but may also be cytokines or of plant origin (saponins).

Oil emulsions are vehicle adjuvants that act as a depot for the antigen resulting in the slow release of the antigen and consequently sustained dendritic cell stimulation over an extended period of time before the host is finally able to clear the antigen. Complete Freund's adjuvant (CFA) is an adjuvant that has, until recently, been commonly used in animal trials (Alving, 2002). It is composed of inactivated *Mycobacterium tuberculosis* emulsified in mineral oil. The incomplete form of this adjuvant (IFA) lacks the mycobacterial component and is now more commonly used in animal trials due to the severe and painful host reactions associated with CFA (Oscherwitz et al., 2006). Both CFA and IFA are potent adjuvants that are able to elicit both Th1 and Th2 cellular immune responses, however both are deemed unsuitable for use in humans due to safety concerns (Brunner et al., 2010). The novel oil adjuvant MontanideTM ISA 206 adjuvant (Seppic, Paris) has been found to improve the stability of vaccine formulations and elicits good antibody responses in both pigs and cattle without the associated toxicity and side effects observed with other adjuvant formulations (Barnett et al., 1996). This adjuvant has been shown to be able to raise better immunity in pigs than aluminium hydroxide Al(OH)₃, a common aluminium-containing adjuvant that has been used to enhance the immune response against inactivated and subunit vaccines for over 70 years (Marrack et al., 2009).

In this study L-Arginine-solubilized VP7-177(P₁)-FMDVepi trimers administered with Montanide™ ISA 206 adjuvant (Barnett et al., 1996) elicited a neutralizing humeral immune response equivalent to that elicited by the same antigen dose in the presence of Complete Freund's Adjuvant. Thus Montanide™ ISA 206 may be considered as a safer alternative to Complete Freund's Adjuvant. The administration of the same dose of L-arginine-solubilized VP7-177(P₁)-FMDVepi trimers with Aluminium hydroxide (Al(OH)₃)/Saponin adjuvant, however, elicited a much weaker neutralizing humeral immune response. Thus, due to its inefficacy, the Al(OH)₃/Saponin combination adjuvant will no longer be considered for use in this study. Another advantage of the Montanide™ ISA 206 adjuvant was that less antigen was required to elicit a good humeral immune response as 20µg of L-arginine-solubilized VP7-177(P₁)-FMDVepi trimers elicited an equivalent immune response to that elicited by 80µg of the same antigen.

Chapter 4

The effect of inserting enhanced green fluorescent protein on AHSV VP7 trimer formation, solubility and folding

4.1 Introduction

The insert-specific, neutralizing antibody immune response elicited by soluble chimeric VP7 trimer molecules (Chapter 3) indicated that an antigenic peptide is presented on the surface of the AHSV VP7 trimer in its native immunogenic conformation. The site of foreign peptide insertion, as well as the inserted peptide, affects the solubility of the VP7 fusion protein (Chapters 2 and 3). Although these factors do not affect trimer formation, they do influence trimer stability. The reduction in trimer stability observed following the insertion of a 150-mer AHSV-4 VP2 peptide into the VP7-177(P₁) vector protein (Chapter 3) led to the current investigation of how the insertion of large peptides/proteins into the top domain of VP7 affect trimer formation and/or stability. It was also not known whether VP7 protein solubility would be affected and how the insertion of these large peptides/proteins into different sites within the VP7 top domain would affect protein folding. To address these questions the Enhanced green fluorescent protein (eGFP) was inserted into sites 144(P₂), 177(P₁) and 200(P₁) within the VP7 top domain (Fig 4.1; Table 2.2).

Enhanced green fluorescent protein (eGFP) is composed of 239 amino acids and has been shown to fluoresce thirty-five times brighter than the wild-type GFP protein (Cormack et al., 1996, Zhang et al., 1996). Green fluorescent protein folding technology was developed as a means of rapidly assessing the correct folding of proteins of interest (Waldo et al., 1999). It was found that the fluorescence of GFP-fusion proteins correlated with the correct folding of the protein of interest expressed alone. A correctly folded protein of interest would not interfere with the correct folding of the fused GFP protein and, consequently, the formation of the fluorescing chromophore within this reporter protein. Thus the intrinsic fluorescence of GFP can be used to assess the folding state of the target protein to which it is fused (Giepmans et al., 2006). In addition, improved folding of a protein also improves its solubility. A number of correctly folded, soluble protein variants have been identified using the GFP folding reporter technology following random mutagenesis and include soluble peptide sequences from the 10th fibronectin type III domain of human fibronectin (10FnIII) (Olson & Roberts, 2007), soluble viral surface protein fragments from the surface glycoproteins of SARS-CoV and HIV-1 (Lin et al., 2009), soluble protein secondary structural elements from *Escherichia coli* (Graziano et al., 2008), identification of insoluble amyloid beta (Aβ) peptides that play a role in Alzheimer's disease (Kim & Hecht, 2005) and identification of a soluble mutant tobacco etch virus (TEV) protease used in the cleavage of recombinant fusion proteins (van den Berg et al., 2006).

It has previously been shown that the insertion of the eGFP protein into site 177(P₁) does not affect VP7 trimer formation (Mizrachi, 2008). Formation of trimers by the VP7-144(P₂)-eGFP and VP7-200(P₁)-eGFP will be investigated in this study. Also investigated will be the stability of any VP7-eGFP trimers formed. The solubility of the VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP proteins will be compared under standardized experimental conditions. The solubility of the VP7-eGFP fusion proteins following L-arginine treatment will be also determined as this solubilization agent has been shown to reverse the aggregation of correctly folded VP7 trimers thereby yielding a more relevant solubility result (Chapter 3). The fluorescence emitted by eGFP protein can be correlated with correct protein folding (Giepmans et al., 2006, Waldo et al., 1999). As the VP7-eGFP proteins have been shown to fluorescence to some degree in previous studies (Mizrachi, 2008) (Semeco, Meyer, unpublished results), it can be concluded that the eGFP protein is correctly folded when inserted into sites 144(P₂), 177(P₁) and 200(P₁). To determine whether the insertion of eGFP into any particular site within the VP7 top domain allows for this protein to fold more easily into its native, functioning conformation, thus yielding a larger number of correctly folded trimers, the ratio of fluorescence emitted per unit soluble protein will also be determined for each of the three VP7-eGFP fusion proteins.

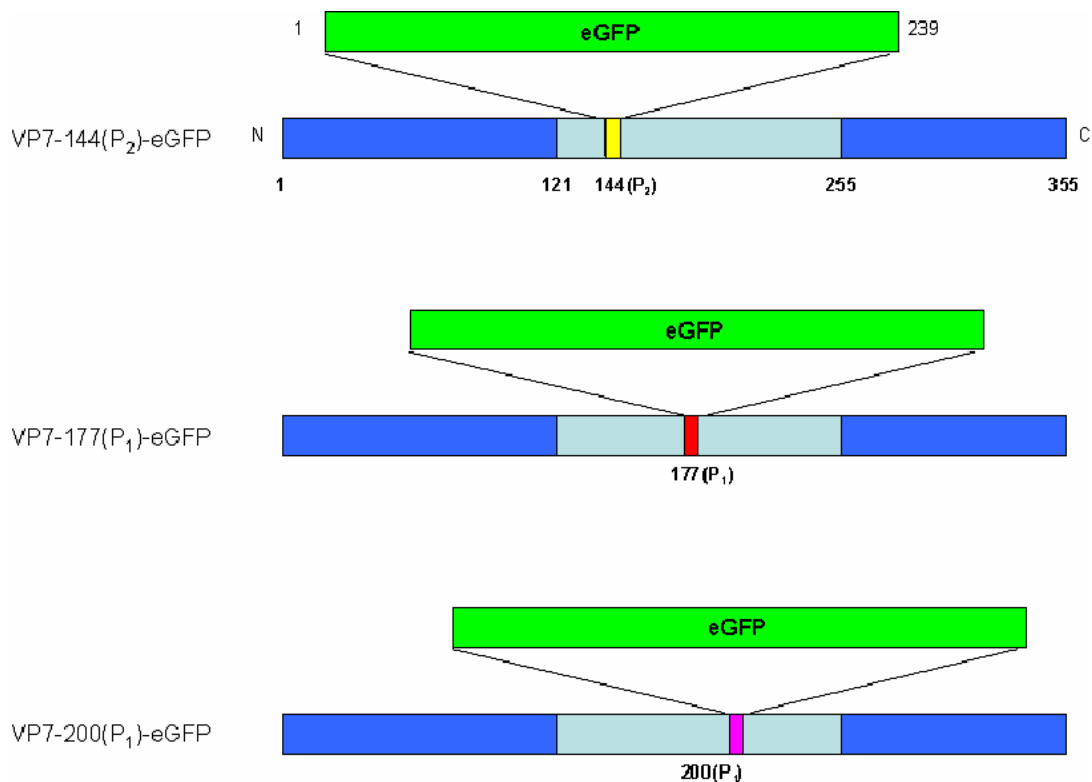


Fig 4.1: A schematic representation of the three VP7-eGFP fusion proteins characterized in this study. The enhanced green fluorescent protein (eGFP) was inserted into sites 144(P₂), 177(P₁) or 200(P₁) within the VP7 vector top domain (amino acids 121-255) as indicated.

4.2 Materials and Methods

4.2.1 Expression and characterization of the VP7-eGFP fusion proteins

Recombinant baculoviruses were used to infect *Spodoptera frugiperda* (Sf9) cells, as described previously (2.2.4), and the cells harvested 38, 46 or 48 hours post-infection. The cell pellets were resuspended in either 0.01M STE buffer or Lysis buffer (0.01M STE, 0.5% Nonidet® P40 (Roche) and, after an incubation period of 30min on ice, homogenized by twenty strokes with a 1ml dounce homogenizer (Wheaton). Solubility analyses of the VP7 fusion proteins were performed by sucrose density gradient centrifugation (2.2.5), the varying conditions of which are documented in each results section. Exposure to UV allowed for the visualization of the fluorescing VP7-eGFP protein bands within the ultracentrifuge tube and the sucrose gradients were fractionated into a number of fractions, the details of which are given in each results section. Following fractionation of the sucrose gradients, the fluorescence ($\lambda=480\text{nm}$) emitted by each sucrose fraction was measured by means of the VersaFluor™ Fluorometer (Biorad), with an excitation filter Ex 490/10 on a medium setting, or the Fluoroskan Ascent FL fluorometer (Thermo Labsystems), using an excitation filter of 485nm and an emission filter of 538nm. The protein content of each sucrose fraction of the gradient was ascertained by 12% SDS-polyacrylamide gel electrophoresis (2.2.6). The identity of the VP7-eGFP fusion proteins was confirmed via immunoblot analysis (2.2.7) with a GFP-specific primary antibody, Rabbit α -GFP-N-terminal (Sigma Life Science). Relative quantification of the VP7-eGFP fusion protein bands on the immunoblot membranes was performed by either utilizing the EZQuant software package (EZQuant Ltd) or by making use of the Image Lab Software Version 2 (BioRad). Trimer formation by the VP7-eGFP fusion proteins was assessed by low density (10-40%) sucrose density sedimentation analysis (2.2.5) and by means of a modified SDS-PAGE protocol, where protein samples were incubated at room temperature in the presence of SDS prior to electrophoresis to confirm the presence of trimers (2.2.6). The relative fluorescence per unit soluble VP7-eGFP protein was calculated by dividing the sum of the fluorescence values emitted by soluble VP7-eGFP protein in specific sucrose fractions by the sum of the relative protein quantification values in those same sucrose fractions.

4.2.2 Strategies to increase the yield of soluble VP7-eGFP fusion protein

Recombinant baculovirus-infected Sf9 cells were harvested 48 hours post-infection and the cell pellet subsequently resuspended in either 0.01M STE buffer or Lysis buffer (0.01M STE with 0.5% Nonidet® P40 (Roche) (2.2.4). If the solubilization agent L-arginine was required to be present prior to cell lysis, 1M L-arginine (Merck) was added to the cells at this stage and the cells incubated for 30 min on ice. The cells were lysed as described in 2.2.4. If particulate VP7 protein was required for treatment with L-Arginine, it was isolated by centrifuging the cellular lysate through a 40% sucrose cushion at 27342 xg (15000 rpm) for 20 min. The particulate protein in the pellet was resuspended in 1xPBS buffer and either treated with 1M L-arginine (Merck) or left untreated. The arginine-treated cellular lysate or arginine-treated VP7 particulate protein was centrifuged

through sucrose density gradients to analyse protein solubility (2.2.5) under the varying centrifugation conditions described in each results section. The visualization of the fluorescing VP7-eGFP protein bands, the measurement of fluorescence emitted by the VP7-eGFP protein in each sucrose fraction and the relative quantity of VP7 protein in each sucrose fraction was assessed as described above (4.2.1).

4.3 Results

4.3.1 Trimer formation of the VP7-eGFP fusion proteins

Soluble fluorescing VP7-177(P₁)-eGFP fusion protein was previously shown to have the sedimentation profile of a VP7-177(P₁)-eGFP trimer (Mizrachi, 2008). In order to determine whether the soluble components of the VP7-144(P₂)-eGFP and VP7-200(P₁)-eGFP fusion proteins were composed of trimers, we compared the sedimentation profiles of these fusion proteins to that of the VP7-177(P₁)-eGFP trimer. The VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP fusion proteins were expressed in Sf9 cells, the cells harvested 48 hours post-infection and subsequently lysed in lysis buffer (0.01M STE, 0.5% Nonidet[®] P40), as described in 4.2.1. Cellular lysates were centrifuged through 10-40% sucrose gradients for 21 hours at 303800 xg (50000 rpm). These centrifugation conditions were selected to ensure optimal separation of soluble VP7 trimers from soluble VP7 dimers or monomers. Exposure to UV illumination allowed for visualization of the fluorescing VP7-eGFP fusion proteins on the sucrose density gradients. The gradients were fractionated into approximately 30 fractions and the fluorescence in the first 13 or first 19 sucrose fractions measured by means of the VersaFluor[™] Fluorometer.

The sedimentation profiles of the soluble, fluorescing VP7-eGFP fusion proteins in sucrose density gradients is shown in Fig 4.2. Under the centrifugation conditions, the soluble VP7-144(P₂)-eGFP and VP7-177(P₁)-eGFP proteins were observed as a single fluorescing band close to the bottom of the tube. The soluble VP7-200(P₁)-eGFP protein was, however, observed as two distinct fluorescing bands within the ultracentrifuge tube. The band near to the bottom of the tube was in the same position as the VP7-144(P₂)-eGFP and VP7-177(P₁)-eGFP bands while the second VP7-200(P₁)-eGFP band was located well above the position of these bands. The amount of fluorescence within the pellets of the gradients also appeared to vary and most likely reflected the different tendencies of the soluble VP7-eGFP proteins to aggregate (discussed later). The fluorescence distribution within the sucrose fractions of gradients was quantified and the result given in Fig 4.3. The fluorescence readings indicated that the majority of the fluorescence emitted by the soluble VP7-144(P₂)-eGFP and VP7-177(P₁)-eGFP proteins was located in sucrose fractions 4-6. Although there was some fluorescence emitted by the VP7-200(P₁)-eGFP protein within these fractions, the majority of the fluorescence was located in sucrose fractions 9-13.

VP7-144(P₂)-eGFP VP7-177(P₁)-eGFP VP7-200(P₁)-eGFP

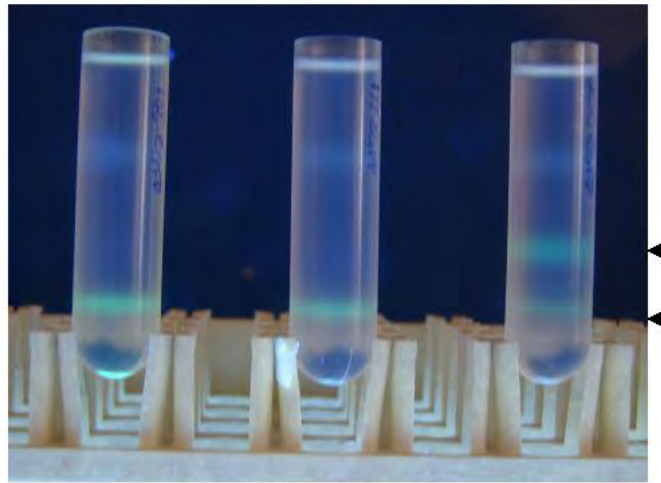


Fig 4.2: Photograph of 10-40% density sucrose gradients, containing fusion proteins, VP7-177(P₁)-eGFP, VP7-144(P₂)-eGFP and VP7-200(P₁)-eGFP, under UV illumination. Cellular lysates were centrifuged for 21 hours at 303800 xg (50000 rpm). Arrows indicate the position of the soluble, fluorescing VP7-eGFP fusion protein within the sucrose gradients.

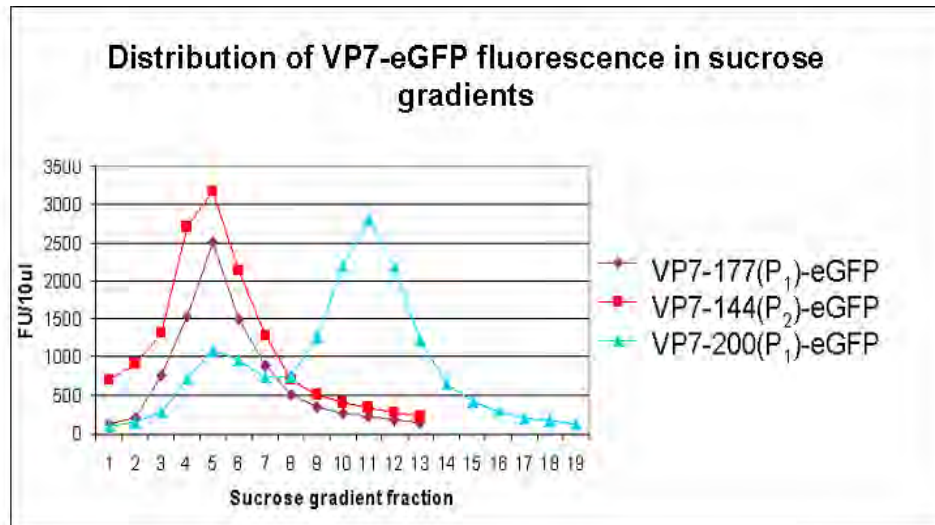


Fig 4.3: Distribution of fluorescence emitted by the soluble VP7-177(P₁)-eGFP, VP7-144(P₂)-eGFP and VP7-200(P₁)-eGFP proteins in fractions 1-13 (1-19 in the case of VP7-200-eGFP) of their respective 10-40% density sucrose gradients. Fraction 1 lies at the bottom of the sucrose gradient while fraction 19 lies close to the top of the gradient. FU indicates fluorescence units.

To confirm that the fluorescing protein in sucrose fractions 4-6 was composed of soluble VP7-eGFP trimers, a modified SDS-PAGE strategy (2.3.3.3., 3.3.1.2) was used. Proteins in fractions 1-13 of the above-mentioned sucrose gradients were subjected to SDS polyacrylamide electrophoresis without prior denaturation at 95°C. The

identity of the VP7-eGFP fusion proteins was established by immunoblotting with a GFP-specific antibody, as described in 4.2.1.

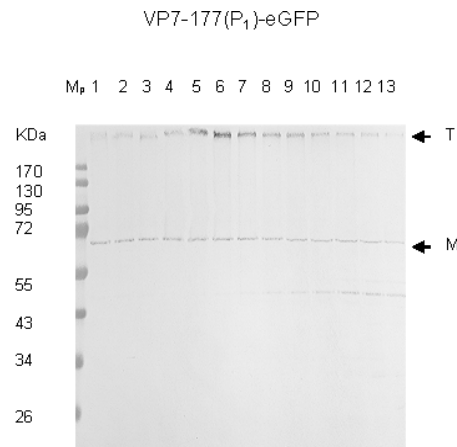


Fig 4.4: Immunoblot analysis to assess the stability of VP7-177(P₁)-eGFP trimers in the presence of SDS at room temperature. Samples from the first 13 fractions of the sucrose gradient containing the VP7-177(P₁)-eGFP protein were incubated at room temperature rather than 95°C prior to 12% SDS-PAGE. The VP7 protein on the immune blot was identified with rabbit α -GFP-N-terminal antibody. The VP7 trimer position (T) and the monomer position (M) are indicated. Marker protein was the PageRuler™ Prestained protein ladder (Fermentas) (M_p). The relevant sizes are indicated in kDa. Lanes 1-13 contain sucrose fractions 1-13 of the sucrose density gradient, respectively.

The soluble VP7-177(P₁)-eGFP protein migrated as both a high molecular weight GFP-specific protein band, visible above the 170KDa protein marker, as well as a 65KDa GFP-specific protein band, on the SDS-polyacrylamide gel (Fig 4.4). As VP7-177(P₁)-eGFP trimers are approximately 195KDa in size, and previous experiments indicated that the high molecular weight VP7-specific protein bands observed in this position were composed of trimer molecules (2.3.3.), the GFP-specific high molecular weight protein band was concluded to be composed of VP7-177(P₁)-eGFP trimers. These results thus confirmed the presence of fluorescing VP7-eGFP trimers in sucrose fractions 4-6 (Fig 4.3). Thus the insertion of the eGFP protein into sites 144(P₂), 177(P₁) and 200(P₁) did not interfere with the trimer formation, as previously shown in the case of VP7-177(P₁)-eGFP (Mizrachi, 2008). The presence of a 65KDa GFP-specific protein band in the same fractions on the SDS polyacrylamide gel, however, indicated that the VP7-177(P₁)-eGFP trimers were less stable in the presence of SDS at room temperature than the VP7-177(P₁) vector trimers (2.3.3.3). This has also been seen with the VP7-177(P₁)-VP2(150aa) trimers (Chapter 3). The complete disassociation of the VP7-144(P₂)-eGFP trimers into monomers during SDS polyacrylamide electrophoresis (result not shown) indicated that the VP7-144(P₂)-eGFP trimers were even less stable than the VP7-177(P₁)-eGFP trimers in SDS at room temperature. A small percentage of the soluble, fluorescing VP7-200(P₁)-eGFP was also present in the form of trimers in sucrose fractions 4-6 (Fig 4.3). However, the majority of this protein sedimented in a position assumed to be that of a monomer and indicated that the VP7-200(P₁)-eGFP trimer is more unstable than the VP7-144(P₂)-eGFP and VP7-177(P₁)-eGFP trimers under normal conditions. The stability of the VP7-eGFP trimers reflected that of the

VP7 vector trimers (2.3.3) and confirmed the influence of the insertion site on VP7 trimer stability. Although VP7 monomers could possibly be effective at eliciting an immune response, only the insertion of a relatively large protein, such as eGFP, into site 200(P₁) would allow VP7 to be present in a monomeric form under normal conditions. This is due to the VP7 trimer being a very stable molecule. The small quantity of soluble, correctly folded VP7-200(P₁)-eGFP monomers that were obtained in this study also casts doubt on the feasibility of a monomeric VP7 presentation system. Next the solubility of the VP7-eGFP fusion proteins was investigated.

4.3.2 Solubility of the VP7-eGFP fusion proteins

The ratio of soluble VP7-177(P₁)-eGFP protein to aggregated VP7-177(P₁)-eGFP protein was shown to decrease with time post-infection (Mizrachi, 2008). To determine how the insertion of eGFP into sites 144(P₂), 177(P₁) and 200(P₁) affected VP7 protein solubility, cells expressing the VP7-eGFP fusion proteins (Table 2.2) were harvested 38 hours post-infection, early in the infection cycle, to ensure the presence of a soluble VP7 fraction. Following resuspension in 0.01M STE buffer, the cells were lysed as described in 4.2.1 and the cellular lysates centrifuged through 30-80% sucrose gradients for 21 hours at 109368 xg (30000 rpm). Gradients were fractionated into 30 fractions and the fluorescence emitted from these fractions, and the pellet fractions, measured by means of the Fluoroskan Ascent FL Fluorometer (Thermo Labsystems) (4.2.1). The protein content of the first 28 sucrose gradient fractions was analysed by 12% SDS-PAGE and VP7-eGFP protein identity confirmed by immunoblotting as described in 4.2.1. The VP7 protein bands were quantified relative to each other by utilizing the EZQuant quantification software (4.2.1).

The protein quantification results indicated that insertion of the eGFP protein into site 144(P₂) resulted in the most soluble VP7-eGFP protein, with approximately 35% of the expressed VP7-144(P₂)-eGFP protein being soluble under the experimental conditions specified (Fig 4.5, fractions 16-25). The VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP fusion proteins were less soluble, with soluble components of about 19.5% and 22%, respectively, in sucrose fractions 16-25. As fluorescence is considered an indicator of correctly folded protein (Giepmans et al., 2006, Waldo et al., 1999), the majority of the correctly folded VP7-eGFP protein was located in soluble sucrose fractions 16-25. The soluble VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP proteins in fractions 16-25 emitted 58%, 55 and 70% of the total fluorescence measured in their respective sucrose gradients. The majority of the VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP proteins, present in sucrose fractions 1-15 and pellet of their respective gradients, emitted little fluorescence. Thus most of the VP7-eGFP protein in these fractions were most likely misfolded and aggregated. In order to determine whether this was indeed the case, the effect of L-arginine treatment on the VP7-eGFP aggregates was investigated. If the aggregates consisted of misfolded protein, mild solubilization agent L-arginine would not be able to solubilize them (Chapter 3).

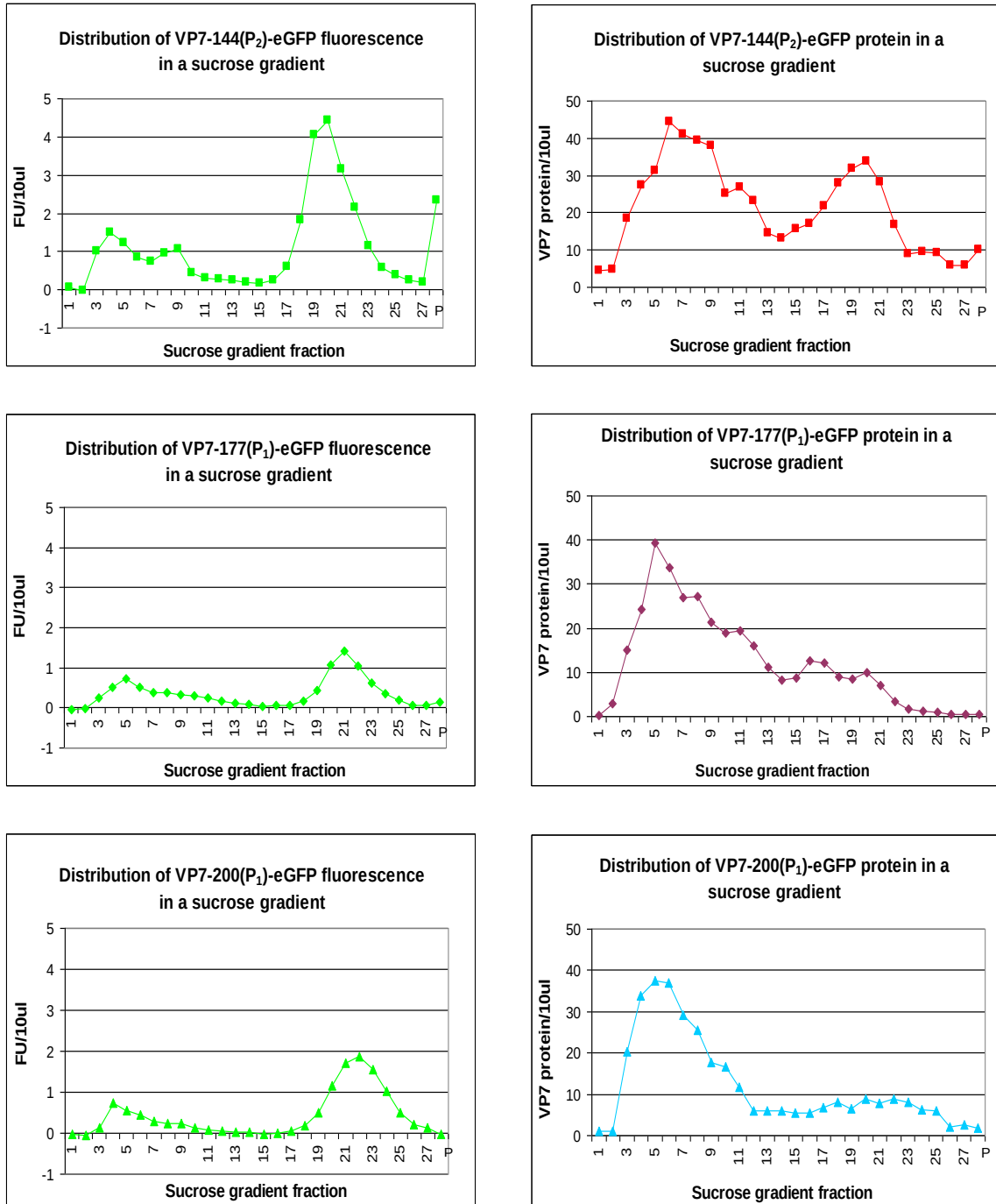


Fig 4.5: Fluorescence and protein distribution of the VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP proteins in their respective 30-80% density sucrose gradients. P indicates the pellet fraction. FU indicates fluorescence units.

4.3.3 Effect of L-arginine treatment on VP7-eGFP fusion protein solubility

The solubilization agent L-arginine is able to reverse aggregation of correctly folded VP7 fusion trimers, but not of misfolded trimers (Chapter 3). To determine whether aggregates formed the VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP proteins are composed of correctly folded trimer molecules, L-arginine was added to the cellular lysate containing these aggregates. More specifically, the VP7-eGFP proteins were expressed in Sf9 insect cells and the cells harvested 48 hours post-infection. Following resuspension in lysis buffer (0.01M STE, 0.5% Nonidet[®] P40), the cells were lysed and the cellular lysates either treated with 1M arginine or left untreated (4.2.1; 4.2.2). The cellular lysates were centrifuged through 50-70% sucrose gradients for 18 hours at 30000 rpm. The gradients were fractionated into 10 fractions and the fluorescence emitted from these sucrose fractions and pellet measured using a Fluoroskan Ascent FL Fluorometer (Thermo Labsystems) (4.2.1).

The fluorescence emitted by the soluble VP7-144(P₂)-eGFP trimers in sucrose fractions 7-9 increased approximately three fold following treatment of the cellular lysate with L-arginine (Fig 4.6). This increase, and the corresponding decrease in the fluorescence emitted by the aggregated VP7-144(P₂)-eGFP protein in the pellet of the gradient, was thought to be due to the release of the correctly folded, fluorescing soluble VP7-144(P₂)-eGFP trimers from the insoluble protein aggregates. Some correctly folded VP7-144(P₂)-eGFP trimers, trapped within a gelatinous precipitate together with misfolded trimers, were responsible for the fluorescence in sucrose fraction 1. No significant increase in the fluorescence emitted by the soluble VP7-177(P₁)-eGFP trimers and VP7-200(P₁)-eGFP trimers/monomers was observed following L-arginine treatment of the cellular lysate. This indicated that the VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP aggregates in fractions 1-15 and pellet shown in Fig 4.5 were most likely composed of misfolded, non-fluorescing trimers.

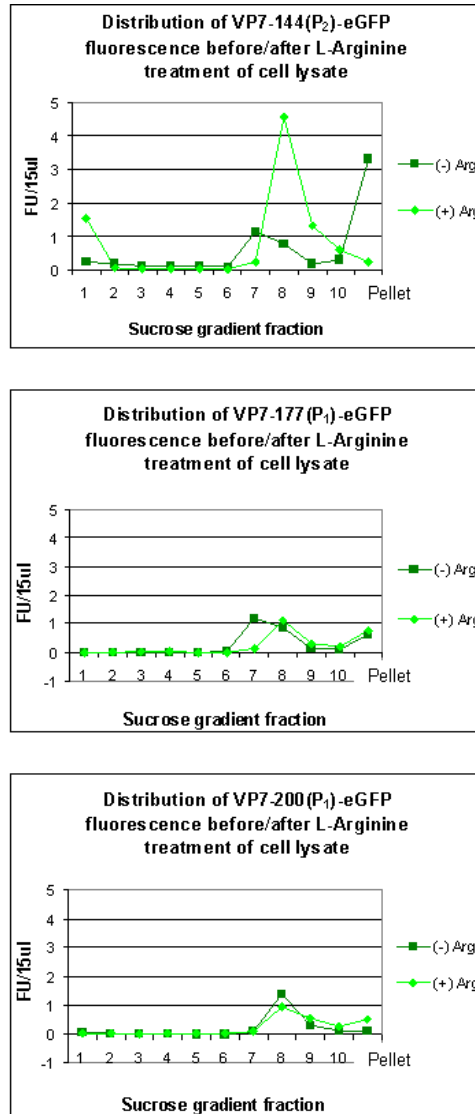


Fig 4.6: Distribution of the fluorescence emitted by the VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP proteins within their respective 50-70% density sucrose gradients following 1M L-Arginine (+Arg) or no L-Arginine (-Arg) treatment of the cell lysate. FU indicates fluorescence units.

To confirm that the increase in fluorescence emitted by the soluble VP7-144(P₂)-eGFP trimers following L-Arginine treatment of the cellular lysate was due to an increase in the amount of soluble VP7-144(P₂)-eGFP protein, this protein was again expressed, harvested and treated as described above. The cellular lysates were centrifuged through 30-80% sucrose gradients for 21 hours at 30000 rpm in order to ensure that both the particulate and soluble VP7-144(P₂)-eGFP protein components were retained on the sucrose gradient. Exposure of the sucrose gradients to UV illumination allowed for visualization of the fluorescing VP7-144(P₂)-eGFP protein bands. The gradients were fractionated into 30 fractions and the fluorescence emitted from these sucrose gradient fractions and pellet measured using a Fluoroskan Ascent FL Fluorometer (Thermo Labsystems) (4.2.1). Sucrose gradient fractions, and pellet fractions, were analysed by 12% SDS-PAGE and VP7-eGFP protein identity

confirmed by immunoblotting, as described in 4.2.1. The VP7 protein bands were quantified relative to each other by utilizing the Image Lab Software (4.2.1).

The sedimentation profiles of the particulate and soluble VP7-144(P₂)-eGFP protein, with and without L-arginine treatment of the cellular lysate, in sucrose density gradients is shown in Fig 4.7. Under the specified centrifugation conditions, the particulate or aggregated VP7-144(P₂)-eGFP trimers were observed as a sharp fluorescing band in the lower half of the gradient while the soluble VP7-144(P₂)-eGFP trimers were observed as a more diffuse fluorescing band located well above the aforementioned band. A significant increase in the fluorescence of the soluble VP7-144(P₂)-eGFP protein band was observed following L-arginine treatment of the cellular lysate. A corresponding decrease in the fluorescence of the aggregated protein band was also observed. The fluorescence and protein distribution was quantified and the result shown in Fig 4.8.

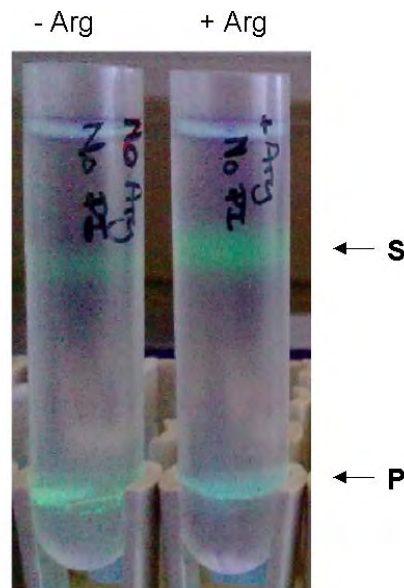


Fig 4.7: Photograph of 30-80% density sucrose gradients, containing the VP7-144(P₂)-eGFP fusion protein, under UV illumination. The cellular lysate, containing this fusion protein, was either treated with 1M arginine (+Arg) or remained untreated (- Arg). Arrows indicate the position of the soluble VP7-144(P₂)-eGFP protein (S) and the particulate VP7-144(P₂)-eGFP protein (P).

Following L-arginine treatment there was a four fold increase of the fluorescence in soluble sucrose fractions 19-27 (Fig 4.8). This corresponded to an equivalent decrease of the fluorescence in fractions 1-18. The amount of soluble VP7-144(P₂)-eGFP trimers in fractions 19-27 was also found to have increased by 30% and was coupled to an equivalent decrease in the amount of aggregated VP7-144(P₂)-eGFP trimers. Thus L-arginine treatment of the cellular lysate reverses VP7-144(P₂)-eGFP trimer aggregation resulting in an increase in the amount of soluble, fluorescing VP7-144(P₂)-eGFP trimers. The fluorescence observed in fractions 1-6 of the sucrose gradient was most likely emitted by some correctly folded, fluorescing trimers that remained trapped with misfolded, non-fluorescing VP7 trimers within an insoluble gelatinous precipitate following L-arginine treatment.

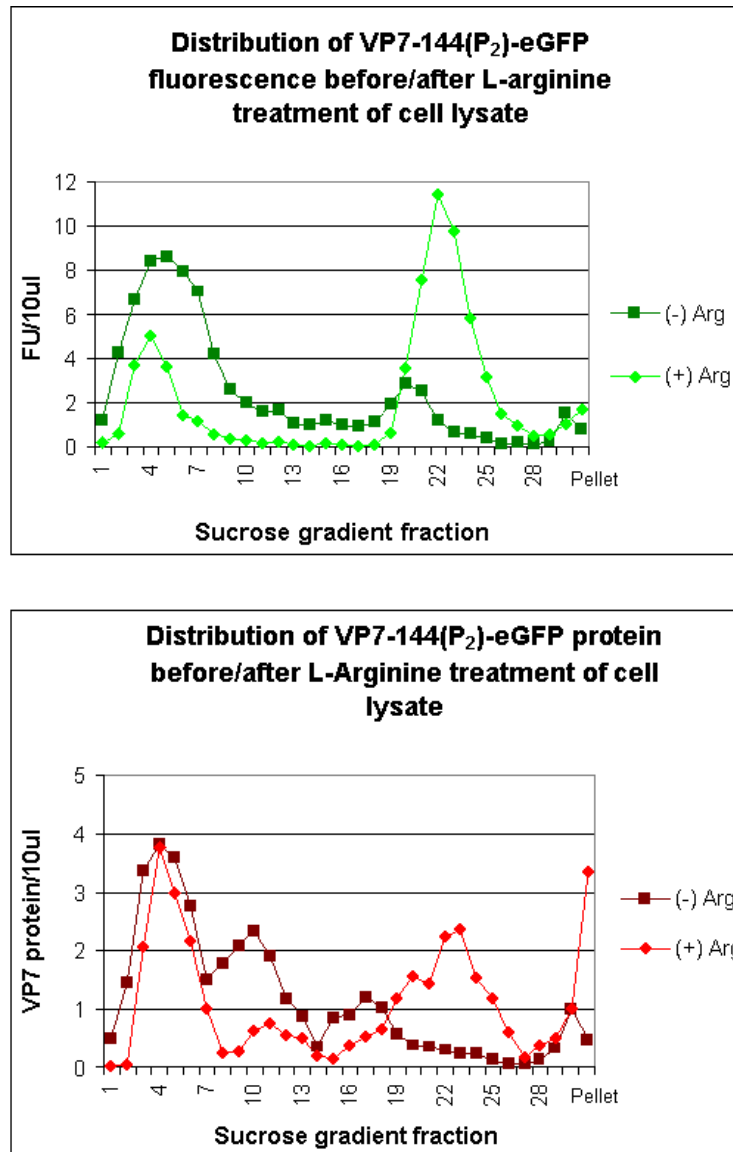


Fig 4.8: Fluorescence and protein distribution of the VP7-144(P₂)-eGFP protein in the 30-80% density sucrose gradients following 1M L-Arginine (+Arg) or no L-Arginine (-Arg) treatment of the cell lysate. FU indicates fluorescence units

It must be noted that the fluorescence and protein distributions of the VP7-144(P₂)-eGFP protein not treated with L-arginine differed significantly in Figures 4.5 and 4.8. The increased amount of soluble VP7-144(P₂)-eGFP protein in Fig 4.5, which was also responsible for the increased fluorescence observed within the soluble fractions, was most likely as a result of the protein being harvested earlier during the infection cycle at 38 hours post-infection. The VP7-144(P₂)-eGFP protein expression levels were thus lower than at 48 hours post-infection (Fig 4.8) and the protein consequently less aggregated.

4.3.4 Degradation of VP7-eGFP protein following L-arginine treatment

In the analysis of the solubility of the VP7-144(P₂)-eGFP protein treated with L-arginine in the cellular lysate (4.3.3), a 42KDa eGFP-specific protein band was observed in addition to the 65KDa VP7-144(P₂)-eGFP protein within the soluble fractions of the sucrose gradient. To determine whether this peptide is the product of partial protease-mediated degradation of the VP7-144(P₂)-eGFP trimer within the cellular lysate, soluble fractions 20-23 of the sucrose gradient obtained in 4.3.3 were combined and centrifuged under conditions that ensured maximum separation of the VP7-144(P₂)-eGFP trimer molecules (195KDa) and any smaller eGFP-containing peptides that may have been present. These conditions included centrifugation through a 10-50% sucrose gradient at 50000 rpm for 21 hours. The gradient was fractionated into 30 fractions and sucrose gradient fractions and pellet fractions analysed by 12% SDS polyacrylamide electrophoresis. The protein samples were denatured at 95°C prior to electrophoresis and the VP7-144(P₂)-eGFP protein identity confirmed by immunoblotting as described in 4.2.1 (Fig 4.9).

The 42KDa eGFP-specific peptide was found to have the same sedimentation profile as the VP7-144(P₂)-eGFP trimer (65KDa). This indicated that this peptide was part of the original trimer during centrifugation and prior to SDS polyacrylamide electrophoresis. Had the cellular proteases cleaved the peptide off the trimer within the cellular lysate, the centrifugation conditions here would have ensured size-based separation of the trimer molecule and the peptide within the sucrose gradient. It is more likely that the cellular proteases introduced site-specific nicks within the trimer molecule that did not affect the sedimentation profile of the VP7 trimers but resulted in partial degradation of the trimer molecule during SDS polyacrylamide electrophoresis. Although only the 42KDa eGFP-specific peptide was observed during this experiment due to the specificity of the eGFP antiserum, it is possible that there were also additional peptides present in sucrose fractions 3-16 following SDS-electrophoresis. The sequence of these peptides is dependent on the sites of the nicks introduced by the cellular proteases which may occur either in the VP7 protein or the eGFP protein. Treatment of the cellular lysate with sarkosyl had previously resulted in the disappearance of the VP7-177(P₁)-FMDVepi protein (3.3.2.3). It is likely that cellular proteases introduced nicks in the VP7-177(P₁)-FMDVepi protein trimer resulting in protein degradation during SDS polyacrylamide electrophoresis.

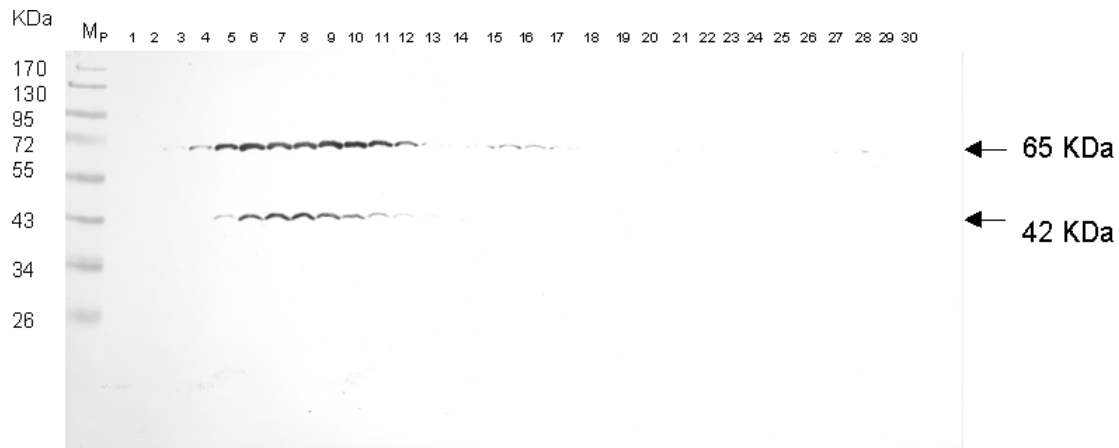


Fig 4.9: Immunoblot analysis to identify the location of eGFP-specific proteins within a 10-50% density sucrose gradient. Soluble VP7-144(P₂)-eGFP protein, treated with L-Arginine within a cellular lysate, was centrifuged at 50000rpm for 21 hours. Samples from sucrose fractions 1-30 were incubated at 95°C prior to 12% SDS-PAGE. eGFP-specific proteins were detected with rabbit α-GFP-N-terminal antibody and are indicated by arrows. Marker protein was the PageRuler™ Prestained protein ladder (Fermentas) (M_P). The relevant sizes are indicated in kDa. Lanes 1-30 contained sucrose fractions 1-30 of the sucrose gradient, respectively.

To investigate the hypothesis that cellular proteases within the cellular lysate are responsible for introducing nicks within the VP7 trimer, an experimental strategy was used that separated the cellular proteases from the VP7-144(P₂)-eGFP trimer aggregates prior to L-arginine solubilization. Aggregated VP7-144(P₂)-eGFP trimers were harvested from the cellular lysate via centrifugation through a 40% sucrose cushion, effectively isolating them from the soluble cellular proteases (4.2.2). The protein aggregates were treated with 1M arginine or left untreated and centrifuged through 30-80% sucrose gradients for 21 hours at 30000 rpm. These centrifugation conditions ensured that both the particulate and soluble VP7-144(P₂)-eGFP protein components remained on the sucrose gradient. The gradients were fractionated into 30 fractions and the fluorescence emitted from these sucrose fractions and pellet measured using a Fluoroskan Ascent FL Fluorometer (Thermo Labsystems) (4.2.1). Sucrose gradient fractions, and pellet fractions, were analysed by 12% SDS-PAGE and VP7-eGFP protein identity confirmed by immunoblotting as described in 4.2.1. The VP7 protein bands were quantified relative to each other by utilizing the Image Lab Software (4.2.1).

The distribution of the fluorescence and the VP7-144(P₂)-eGFP protein on the sucrose gradients, before and after L-arginine treatment of the VP7-144(P₂)-eGFP aggregates, is shown in Fig 4.10. Like L-arginine treatment of VP7-144(P₂)-eGFP aggregates within the cellular lysate, a significant increase in the amount of soluble, fluorescing VP7-144(P₂)-eGFP trimers was observed following L-arginine treatment. However, unlike arginine treatment of the cellular lysate, no additional 43kDa eGFP-specific band was observed after electrophoretic analysis of the soluble proteins in fractions 18-26 of the sucrose gradient following treatment of the VP7-144(P₂)-eGFP aggregates with L-arginine (Fig 4.11). This result indicated that the removal of cellular proteases prior to L-arginine treatment of the VP7-144(P₂)-eGFP aggregates prevented the introduction of site-specific nicks within the trimer molecule and the subsequent cleaving of specific peptides off the VP7 trimer under harsh denaturing

conditions. Thus this strategy may be used to ensure that no nicks are introduced into the VP7 fusion trimer and the integrity of the trimer is conserved.

The effect of eGFP insertion into the VP7 top domain on this proteins trimer formation, stability and solubility has thus far been investigated in this study. Although the VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP proteins have all been shown to fluorescence, indicating correct protein folding, it was not known whether insertion into any particular site allowed for the eGFP protein to fold more easily into its native, functional conformation and will be investigated in the next section.

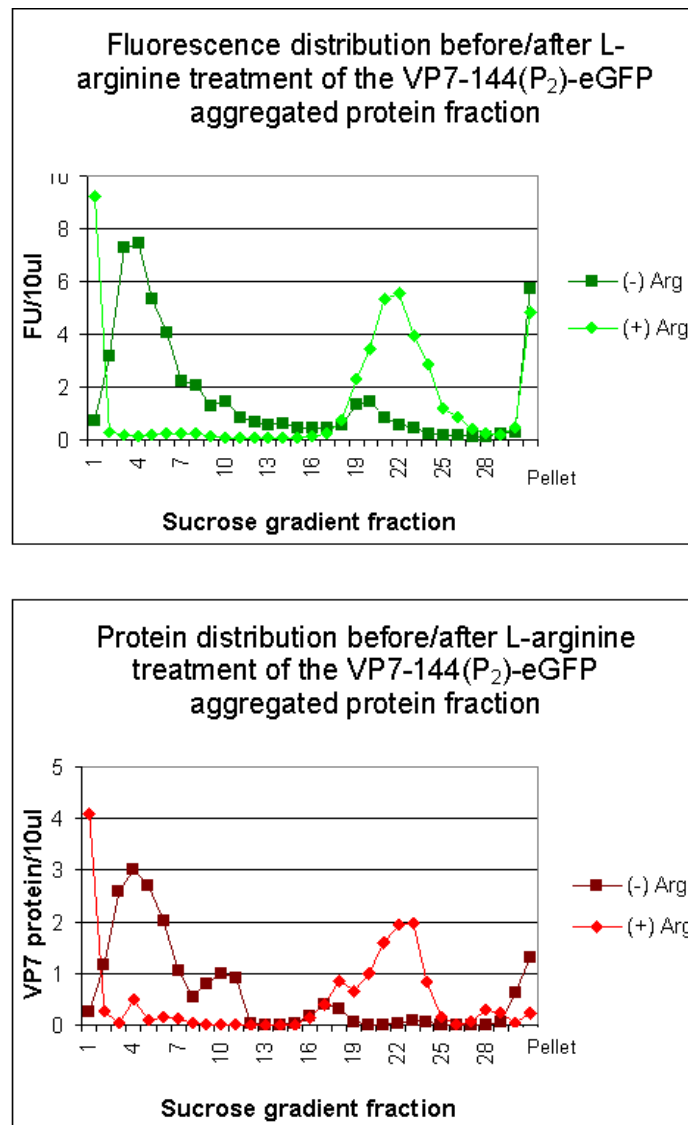


Fig 4.10: Fluorescence and protein distribution of the VP7-144(P₂)-eGFP protein in 30-80% density sucrose gradients following 1M L-Arginine (+Arg) or no L-Arginine (-Arg) treatment of insoluble VP7-144(P₂)-eGFP protein aggregates. FU indicates fluorescence units.

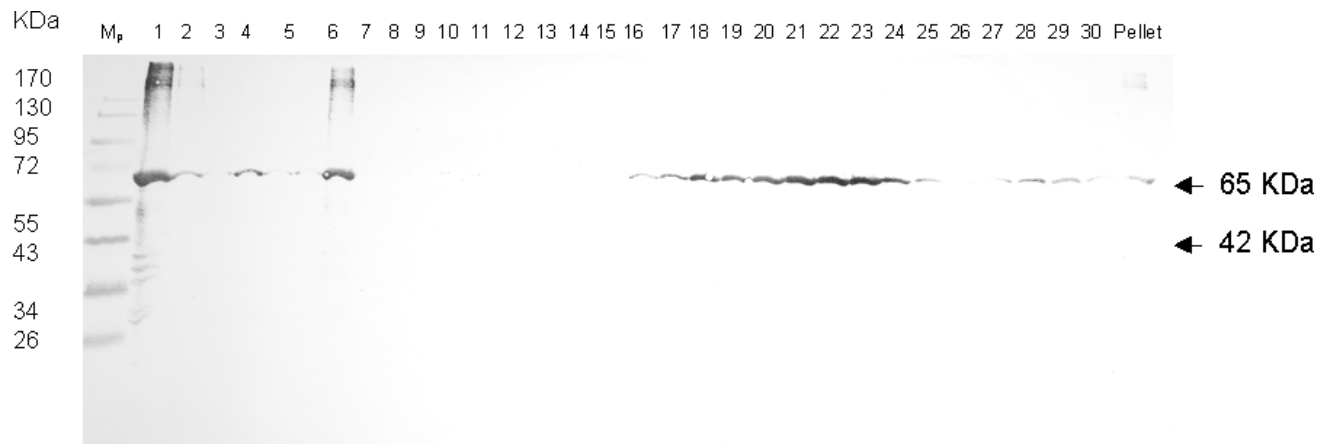


Fig 4.11: Immunoblot analysis to determine the presence of a 42 KDa eGFP-specific protein within a 30-80% density sucrose gradient. VP7-144(P₂)-eGFP (65KDa) protein aggregates were solubilized with L-Arginine and centrifuged through the gradient at 30000rpm for 21 hours. eGFP-specific proteins were detected with rabbit α -GFP-N-terminal antibody and their position is indicated by arrows. Lanes 1-30 contain sucrose fractions 1-30 of the sucrose gradient, respectively. The sucrose fractions were incubated at 95°C prior to 12% SDS-PAGE. Marker protein was the PageRuler™ Prestained protein ladder (Fermentas) (M_p). The relevant sizes are indicates in KDa.

4.3.5 Effect of VP7 insertion site on eGFP protein folding

To determine whether insertion of the eGFP protein into any particular site within the VP7 top domain favoured the formation of correctly folded trimers, the fluorescence emitted per unit soluble protein was calculated for each of the three VP7-eGFP fusion proteins. The VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP proteins were expressed in Sf9 insect cells, harvested 46 hours post-infection and subjected to centrifugation conditions that ensured separation of the VP7 trimers from VP7 monomers, as previously described (4.3.1). Fluorescence readings were taken with a Fluoroskan Ascent FL Fluorometer (Thermo Labsystems) (4.2.1).

The fluorescence emitted by the soluble VP7-144(P₂)-eGFP and VP7-177(P₁)-eGFP proteins peaked in sucrose fractions 7-9 (Fig 4.12). Although a component of the soluble, fluorescing VP7-200(P₁)-eGFP protein was also located within these fractions, fluorescence emitted by the soluble VP7-200(P₁)-eGFP protein peaked in fractions 11-14. Earlier results (4.3.1) lead us to conclude that the VP7-eGFP protein in fractions 7-9 was composed of correctly folded, fluorescing VP7 trimers while the VP7-200(P₁)-eGFP protein in fractions 11-14 was in a fluorescing, monomer form. The increased fluorescence emitted by the soluble VP7-144(P₂)-eGFP protein in sucrose fractions 1-6, when compared to the fluorescence emitted by the VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP proteins within these same fractions, was most likely due to the aggregation of correctly folded, fluorescing VP7-144(P₂)-eGFP trimers.

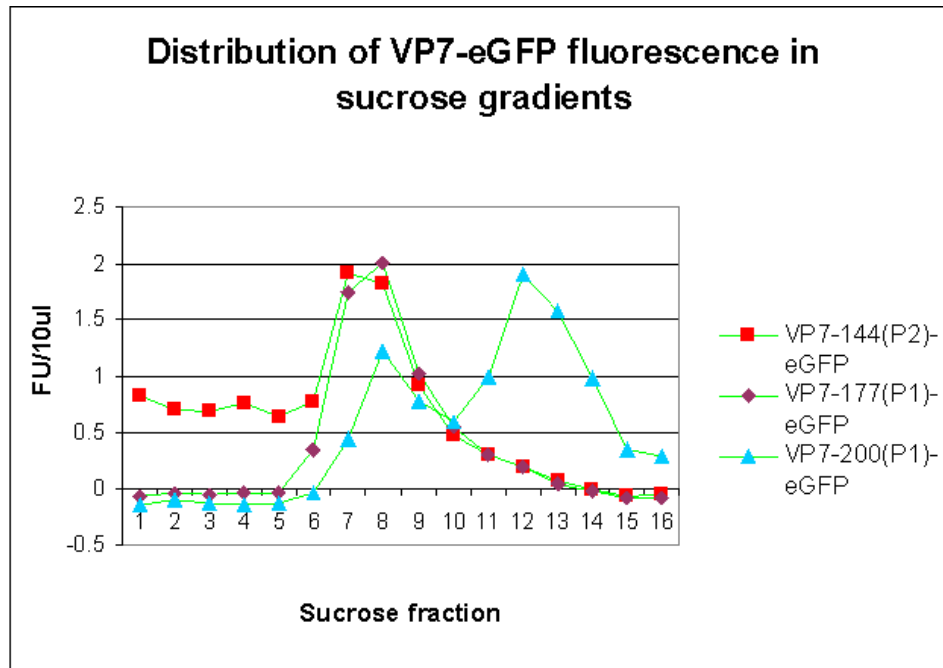


Fig 4.12: Distribution of the fluorescence emitted by the soluble VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP fusion proteins in fractions 1-16 of their respective 10-50% density sucrose gradients. FU indicates fluorescence units.

As the peak of the fluorescence emitted by the soluble VP7-144(P₂)-eGFP and VP7-177(P₁)-eGFP proteins, and a significant amount of the fluorescence emitted by the soluble VP7-200(P₁)-eGFP protein, was located in fractions 7-9 of their respective sucrose gradients, these fractions were subjected to electrophoresis on the same 12% SDS-polyacrylamide gel, followed by immunoblotting with an GFP-specific antibody (4.2.1) (Fig 4.12). Sucrose fractions 10-14, which contained the peak of the fluorescence emitted by the soluble VP7-200(P₁)-eGFP protein, were also included on this gel. The VP7-eGFP protein bands were subsequently quantified relative to each other by utilizing the ImageLab Software - Version 2 (Bio-Rad) (4.2.1). In order to calculate the ratio of fluorescence emitted per unit soluble VP7-eGFP trimer, the sum of the relative fluorescence emitted by the VP7-eGFP trimer in sucrose fractions 7-9 of the sucrose gradient was divided by the sum of the relative quantity of VP7-eGFP protein within these fractions. The ratio of fluorescence emitted per unit soluble VP7-200(P₁)-eGFP monomer was calculated by dividing the sum of the relative fluorescence emitted by the VP7-200(P₁)-eGFP monomer in sucrose fractions 10-14 by the sum of the relative quantity of VP7-200(P₁)-eGFP protein within these fractions. The comparison of these relative ratios indicated that eGFP is able to fold equally well into its functional conformation when inserted into each of the insertion sites within the VP7 top domain (Table 4.1). The variation observed was not considered significant and most likely due to small differences in ratios of soluble correctly folded trimers to soluble misfolded trimers. The VP7-200(P₁)-eGFP monomers fluoresced 23% better than VP7-200(P₁)-eGFP trimers per unit protein. This may indicate that trimer assembly affected the folding of the eGFP protein to a small degree via steric hindrance.

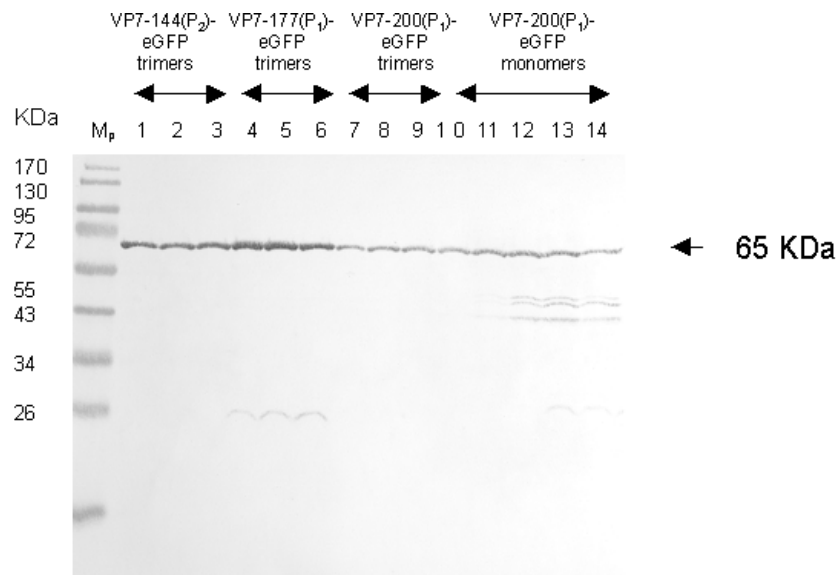


Fig 4.13: Immunoblot analysis to enable relative protein quantification of soluble VP7-eGFP (65kDa). Sucrose gradient fractions 7, 8 and 9, corresponding to the position of the VP7-eGFP trimer, were located in lanes 1-3 (VP7-144(P₂)-eGFP), lanes 4-6 (VP7-177(P₁)-eGFP) and lanes 7-9 (VP7-200(P₁)-eGFP). Sucrose gradient fractions 10-14, corresponding to the position of the VP7-200(P₁)-eGFP monomer were located in lanes 10-14. All samples were all subjected to 12% SDS electrophoresis and immunoblotting with rabbit α -GFP-N-terminal antibody. The position of the VP7-eGFP protein on the membrane is indicated by the arrow. Marker protein was the PageRuler™ Prestained protein ladder (Fermentas) (M_p). The relevant sizes are indicates in kDa.

Table 4.1: Relative ratios of fluorescence per unit soluble VP7-eGFP protein

VP7-eGFP fusion protein	Fluorescence per unit soluble protein
VP7-144(P ₂)-eGFP	1.583352 (trimers)
VP7-177(P ₁)-eGFP	1.096892 (trimers)
VP7-200(P ₁)-eGFP	1.639461 (trimers) 2.130477 (monomers)

4.4 Discussion

The insertion of peptides of up to 150 amino acids in length into the AHSV VP7 top domain does not interfere with the ability of this protein to assemble into trimers (Chapter 3). The stability of these VP7 fusion protein trimers under harsh denaturing conditions is, however, determined mainly by the site of insertion. Although the peptide insert has less of an influence, the insertion of the AHSV-4 VP2 150aa peptide into site 177(P₁) resulted in a decrease in VP7 trimer stability. This result prompted an investigation into whether there is a limit on the size of the peptide that can be inserted into the VP7 top domain without interfering with the ability of the VP7 protein to form trimers. The 239 amino acid enhanced Green Fluorescent Protein (Cormack et al., 1996, Zhang et al., 1996) was subsequently inserted into sites 144(P₂), 177(P₁) and 200(P₁) within the VP7 top domain (Table 2.2) and its effect on VP7 trimer formation and stability investigated. Also investigated was the solubility of these VP7-eGFP proteins under the same experimental conditions. Although the VP7-eGFP proteins had previously been found to fluoresce (Mizrachi, 2008, Meyer, Semeco), indicating correct eGFP protein folding to some degree (Giepmans et al., 2006, Waldo et al., 1999), a comparison of protein folding and conformation following insertion of the same sequence into each of the three insertion sites within the VP7 top domain was lacking. All these questions were addressed in this study.

Soluble, fluorescing VP7-177(P₁)-eGFP fusion protein was previously shown to have the sedimentation profile expected of a VP7-eGFP trimer molecule (Mizrachi, 2008). Although, when compared to that of the VP7-177(P₁)-eGFP trimer, the soluble, fluorescing VP7-144(P₂)-eGFP protein also had the sedimentation profile of a trimer molecule, the soluble, fluorescing VP7-200(P₁)-eGFP fusion protein had the sedimentation profile of both a trimer molecule as well as a monomer following a modified density gradient sedimentation protocol (Limn & Roy, 2003, Limn et al., 2000). VP7-eGFP trimers are hypothesized to be in a state of equilibrium with VP7-eGFP monomers. Results indicated that, in the case of the VP7-177(P₁)-eGFP and VP7-144(P₂)-eGFP proteins, this equilibrium is shifted towards the trimer state while in the case of the VP7-200(P₁)-eGFP protein the equilibrium is shifted towards the monomeric state under normal conditions. Although eGFP insertion into sites 177(P₁) and 144(P₂) does not interfere with the ability of the VP7-177(P₁)-eGFP and VP7-144(P₂)-eGFP monomers to form trimers, insertion into site 200(P₁) interferes with VP7-200(P₁)-eGFP trimer formation and the majority of this protein remains in a monomeric form. Although the N- and C-terminals within the bottom domain of the VP7 protein are primarily responsible for trimer formation (Hosamani et al., 2010, Monastyrskaya et al., 1997, Wang et al., 1994), the VP7 top domain has also been found to play a role (Grimes et al., 1995, Grimes et al., 1998). It is thus not surprising that the insertion of the eGFP protein into the VP7 top domain would affect trimer formation. The decreased ability of the VP7-200(P₁)-eGFP to form trimers may be due to the close spatial proximity of the three 200(P₁) insertion sites within the VP7 trimer (Fig 1.5B). This is the first report of a modification within the VP7 top domain interfering with trimer formation. Although the VP7-200(P₁)-eGFP trimer molecule was clearly less stable than the VP7-144(P₂)-eGFP and VP7-177(P₁)-eGFP trimer under normal centrifugation conditions, harsh denaturing conditions were required to establish the difference in stability

between the VP7-144(P₂)-eGFP and VP7-177(P₁)-eGFP trimers. The VP7-177(P₁)-eGFP trimer disassociated partially into monomers while the VP7-144(P₂)-eGFP trimer disassociated completely into monomers following a previously described, modified SDS-PAGE protocol (Monastyrskaya et al., 1997). Although the stability of the VP7-177(P₁)-eGFP and VP7-144(P₂)-eGFP trimers in the presence of SDS at room temperature reflected that of their respective VP7 vector trimers, with the VP7-177(P₁)-eGFP trimer being more stable than the VP7-144(P₂)-eGFP trimer, the insertion of the eGFP protein into these sites resulted in the fusion trimers being less stable than the vector trimers. These results indicated that, in addition to the influence of the insertion site on VP7 trimer stability, the inserted peptide also plays a role. The peptide inserts' influence on trimer stability was previously seen following the insertion of the AHSV-4 VP2 150aa peptide into site 177(P₁) (Chapter 3).

The solubility of the VP7 fusion protein has also been shown to be influenced by the insertion site as well as the peptide insert (Chapter 3). The effect of inserting a relatively large protein, such as the eGFP protein, into different sites within the VP7 top domain on VP7 protein solubility was investigated under standardized experimental conditions. As it has been shown that the ratio of soluble VP7-177(P₁)-eGFP protein to aggregated VP7-177(P₁)-eGFP protein decreases with time post-infection (Mizrachi, 2008), it was hypothesized that harvesting the VP7-144(P₂)-eGFP, VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP early in the cell infection cycle, at 38 hours post-infection, would ensure the presence of a detectable VP7-eGFP soluble fraction. The VP7-144(P₂)-eGFP protein was the most soluble VP7-eGFP protein, with approximately 35% of the expressed VP7-144(P₂)-eGFP protein being soluble under the experimental conditions specified. The VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP fusion proteins were less soluble, having soluble components of 19.5% and 22%, respectively. As fluorescence is considered an indicator of correctly folded protein (Giepmans et al., 2006, Waldo et al., 1999), the correctly folded VP7-eGFP trimers within these soluble fractions emitted the bulk of the fluorescence observed. However, not all the correctly folded, fluorescing protein was found in the soluble fraction. The insoluble VP7-eGFP aggregates also emitted a degree of fluorescence indicating the presence of a small amount of correctly folded, fluorescing VP7-eGFP trimers amongst the bulk of misfolded, non-fluorescing trimers. As treatment with the solubilization agent L-arginine had previously been found to reverse the aggregation of correctly folded trimers (Chapter 3), the cellular lysate was treated with L-arginine. This allowed us to determine the quantity of correctly folded, fluorescing VP7-eGFP trimers present within the insoluble aggregates, and thus a more accurate estimate of VP7-eGFP protein solubility.

The addition of L-arginine to the cellular lysate, containing the expressed VP7-144(P₂)-eGFP protein, resulted in a significant increase in the amount of soluble, correctly folded and fluorescing VP7-144(P₂)-eGFP trimers. No substantial increase was observed following L-arginine treatment of the VP7-177(P₁)-eGFP and VP7-200(P₁)-eGFP proteins. This result implies that the VP7-177(P₁)-eGFP or VP7-200(P₁)-eGFP aggregates contain few correctly folded, fluorescing trimers that are available for solubilization via L-arginine treatment. In contrast, the VP7-144(P₂)-eGFP aggregates have a significant percentage of correctly folded, fluorescing trimers that can be solubilized by L-arginine treatment. The observed tendency of the correctly folded, fluorescing VP7-144(P₂)-

eGFP trimers to aggregate may either be a characteristic unique to the 144(P₂) insertion site or may simply be due to the overexpression of this soluble protein late in the infection cycle.

Although VP7-144(P₂)-eGFP trimer aggregation could be reversed, L-arginine treatment of the cellular lysate allowed the introduction of site-specific nicks within the VP7 fusion trimer. Although these nicks did not affect the sedimentation profile of the VP7 fusion trimer, specific peptides were cleaved off the trimer under harsh denaturing conditions during SDS polyacrylamide electrophoresis. This phenomenon was also observed following treatment of the VP7-177(P₁)-FMDVepi trimer aggregates with the detergent sarkosyl within the cellular lysate (Chapter 3). Soluble proteases, present in the cellular lysate, were deemed responsible and, indeed, removal of the VP7 aggregates from the soluble cellular proteins, including the proteases, prior to arginine treatment prevented the introduction of these site-specific nicks within the VP7 trimer molecule. Thus the release of soluble, correctly folded trimers from aggregates via L-arginine treatment of the cellular lysate, or soluble correctly folded and incorrectly folded trimers via sarkosyl treatment, may allow cellular proteases greater access to the trimer molecules and enable site-specific nicks to occur. It is likely that the greater the efficacy of aggregate solubilization, such as with the harsher solubilization agent sarkosyl, the greater the amount of trimers nicked by the cellular proteases.

The removal of VP7 trimer aggregates from the soluble cellular environment, prior to L-arginine treatment, not only ensured the removal of the soluble proteases but also the other soluble cellular and viral proteins present in the cellular lysate. This simple low speed centrifugation strategy, followed by L-arginine treatment, thus allows for the harvesting of large quantities of soluble, correctly-folded VP7 trimers that are free from the bulk of cellular and viral proteins, an important criterion for vaccine development. This strategy can, however, only be successfully implemented if the VP7 fusion protein forms correctly folded trimer molecules that aggregate.

The VP7-177(P₁)-eGFP, VP7-144(P₂)-eGFP and VP7-200(P₁)-eGFP proteins have all been found to fluorescence in this and in previous studies (Mizrachi, 2008) (Semeco, Meyer). As fluorescence has been correlated with correct protein folding (Giepmans et al., 2006, Waldo et al., 1999), these studies indicated that the eGFP protein was correctly folded when inserted into sites 177(P₁), 144(P₂) and 200(P₁) of the VP7 top domain. It was, however, not known whether insertion of the eGFP protein into any one specific site would allow the protein to fold more easily into its native functioning conformation, thus resulting in a greater number of correctly folded VP7 trimers. To investigate the fluorescence per unit soluble protein ratio was calculated for each of the VP7-eGFP fusion proteins. The comparison of these relative ratios indicated that the eGFP protein is able to fold equally well into its functional conformation when inserted into sites 177(P₁), 144(P₂) or 200(P₁). Thus the number of correctly folded trimers formed by each of the VP7-eGFP proteins seemed to be equivalent. The small variation observed between the ratios may have been due to the possible presence of soluble, misfolded trimers. As the solubilization agent sarkosyl solubilizes both correctly folded as well as incorrectly folded trimers (Chapter 3), it is not impossible that the milder solubilization agent L-arginine may also solubilize

some incorrectly folded VP7-eGFP trimers. Interestingly, the fluorescence emitted per unit soluble VP7-200(P₁)-eGFP monomeric protein was greater than the fluorescence emitted per unit soluble VP7-200(P₁)-eGFP trimer. This may indicate that the folding, and thus functioning, of the eGFP protein may be affected to a small degree via steric hindrance within the VP7 trimer molecule and needs to be further investigated.

Chapter 5

Use of insoluble VP7-HIV fusion particles in HIV antiserum characterization

5.1 Introduction

The VP7-HIV fusion proteins, constructed in this and in previous studies (Meyer, 2002, Rutkowska, 2002, Van Rensburg, 2004) were found to be predominantly insoluble (Chapter 3) and could not be solubilized by L-Arginine or other treatments (Result not shown). These largely insoluble proteins were, however, expressed in good yields and were suggested as a means of analysing the immune response against small HIV-1 peptides or epitopes in HIV-1 infected patients. There is a need to compare the humeral immune response in different patients and especially in the diagnostic analysis of patients that are exposed to HIV-1 subtype C. Here it will be explored if non-conformational antibodies from HIV-1 subtype C infected patients recognise linear HIV-1 subtype B or subtype C sequences from the membrane proximal external region (MPER) of the gp41 protein. If this is the case then it will be determined whether any differences exist in the binding profiles of the antisera investigated.

The high degree of genetic diversity within the HIV Env protein has resulted in the grouping of HIV-1 isolates in three groups and numerous subtypes (McCutchan, 2000). However, despite this variation, monoclonal antibodies have been isolated from individuals infected with HIV-1 group M subtype B that were able to neutralize primary HIV-1 isolates of homologous, as well as heterologous, subtypes (Kitabwalla et al., 2003, Kliks et al., 1994, Purtscher et al., 1996, Trkola et al., 1995, Verrier et al., 2001, Xu et al., 2001). Two of these antibodies bind to the linear ELDKWA (aa 669-674) (Dong et al., 2001, Muster et al., 1993) and GCSGKLICTT (aa602-611) (Cotropia et al., 1996, Viveros et al., 2000) epitopes located in the MPER region. Also located in the MPER region is the protective linear RILAVERYLKD epitope (aa586-596) (Chen & Dierich, 1998, Dong et al., 2001, Dorn et al., 2000) that may also have cross-subtype neutralizing activity. As HIV-1 subtype C is the most prevalent HIV-1 subtype in sub-Saharan Africa (McCutchan, 2006), subtype B ELDKWA (aa 655-660), RILAVERYLKD (aa 572-582) epitopes, as well as the subtype C ALDSWK (aa 643-648) and RVLAIERLYLKD (aa 560-570) sequences equivalent to these epitopes (Fig 5.1), were inserted into site 177(P₁) or 177(P₃) of the VP7 vector protein (Table 2.2). Also inserted into this site was the subtype C sequence LGLWGCSGKIIC (aa 574-585), containing amino acids equivalent to the HIV-1 subtype B GCSGKLICTT epitope (aa 590-599). These epitopes, or epitope-equivalent regions, were presented with or without the amino acids flanking these domains within the gp41 protein as it was initially hypothesized that the addition of these flanking amino acids would improve epitope display on the surface of the soluble VP7 trimer. Also in an attempt to improve chimeric VP7 protein solubility the subtype C sequence LGLWGCSGKIIC (aa 574-585) sequence was presented with highly hydrophilic arginine residues flanking its N and C terminals. This strategy was hypothesized to increase the

hydrophilic character of the inserted peptide and thus improve protein solubility (Bianchi et al., 1994, Mosavi & Peng, 2003). It was of interest to determine whether HIV-1 subtype C antisera would detect the HIV-1 subtype B epitopes or epitope-equivalent subtype C sequences presented by the VP7 protein and whether the antibodies would also recognise the regions flanking these sequences within the conserved, immunologically important MPER region of the gp41 protein.

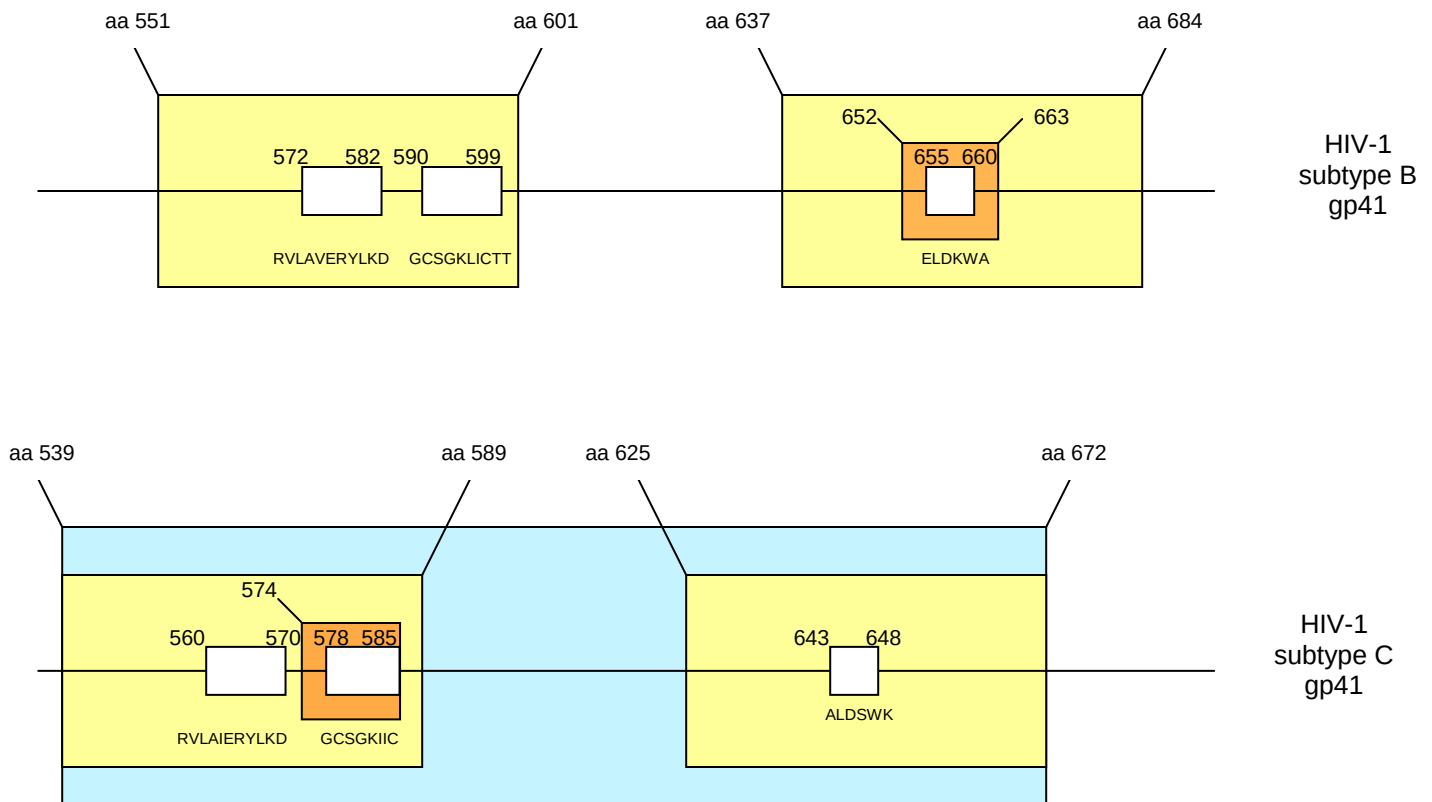


Fig 5.1: A schematic representation of epitopes, and epitope-equivalent domains, located on the gp41 protein of HIV-1 subtypes B and C, respectively. The HIV-1 subtype B epitopes ELDKWA (Dong et al., 2001, Muster et al., 1993), RILAVERYLKD (Chen & Dierich, 1998, Dong et al., 2001) and GCSGKLICTT (Cotropia et al., 1996, Viveros et al., 2000), as well as the corresponding HIV-1 subtype C domains, are indicated in white. Indicated in orange, yellow and blue are the regions, containing these epitopes or epitope-equivalent domains, inserted into the VP7 top domain by various authors (Table 2.2). The HIV-1 subtype C RVLAIERYLKD sequence (aa560-570) was also inserted into the VP7 top domain without flanking amino acids, either as a single copy or as a quadruple repeat (Van Rensburg, 2004).

5.2 Materials and methods

5.2.1 Expression and characterization of VP7-HIV fusion proteins

VP7-HIV fusion proteins VP7-177(P₁)-ELDKWA, VP7-144(P₂)-177(P₃)-RVLA-200(P₁), VP7-144(P₂)-177(P₃)-(RVLA)₄-200(P₁), VP7-177(P₁)-f₁ALDSWKf₂, VP7-177(P₁)-f₃ELDKWaf₄, VP7-177(P₁)-f₅RVLAf₆, VP7-177(P₁)-f₇RILAf₈, VP7-177(P₁)-f₅RVLAf₆f₁ALDSWKf₂, VP7-177(P₁)-RCL3R and the VP7-177(P₁) vector protein, the origin and detail of which is given in Table 2.2, were expressed in recombinant baculovirus-infected *Spodoptera frugiperda* (Sf9) cells and harvested 72 hours post infection, as described previously (2.2.4). The cell pellet was subsequently resuspended in 1000µl Lysis buffer (0.01M STE with 0.5% Nonidet[®] P40 (Roche) and incubated for 30 min on ice. Cellular lysate containing the desired protein was obtained following homogenization of the cell suspension by 20 strokes with a 1ml dounce homogenizer (Wheaton). The cellular lysate, containing VP7 fusion protein, was centrifuged through a 40% sucrose cushion at 27342 xg (15000rpm) for 20min at 4°C. The supernatant was discarded and the particulate component of the VP7 fusion protein, which had centrifuged through into the pellet, resuspended in 1xPBS buffer. The protein content of the pellets was analyzed by 12% SDS-polyacrylamide gel electrophoresis (2.2.6). To characterize human anti-HIV sera, replicas of SDS-PAGE gels were subjected to immunoblot analysis (2.2.7) with the six HIV-1 subtype C antisera listed in Table 5.1.

Table 5.1: The HIV-1 subtype C antisera characterised in this study via immunoblot analysis

HIV-1 subtype C antisera used in Immunoblotting procedures*
17521658/C BB22 Plasma Dec 2004 (Oct 2004) 07/03/05
17487496/C BB14 Plasma Dec 2004 (Oct 2004) 30/06/05
17271157/C BB34 Plasma Dec 2004 (Oct 2004) 28/06/05
17534264/C BB105 Plasma Dec 2004 (Oct 2004) 26/05/05
17193016/C BB81 Plasma Jan 2005 (Oct 2004) 23/06/05
IBU-3 Plasma 22/11/00

* The human HIV-1 subtype C antisera were provided by Dr P. Moore of the National Centre for Infectious Diseases (NCID)

5.3 Results

5.3.1 The use of insoluble VP7 fusion particles in serum characterization

Preliminary evidence indicated that antibodies from a HIV-1 subtype C infected patient were able to recognize and bind all the different VP7-HIV fusion proteins in an immunoblot assay, with the exception of the VP7-177(P₁)-f₃ELDKWAF₄ protein which contained a HIV-1 subtype B sequence (result not shown). This suggested that AHSV VP7 fusions, with a range of different HIV-1 gp41 associated peptides inserted into the VP7 top domain, can be used in immune blots to analyze HIV patient sera and be used to diagnose HIV infection. It also may allow a clear distinction between HIV-1 subtype B and C infected individuals. In order to investigate this possibility further the binding profiles of six antisera, originating from different HIV-1 subtype C infected individuals, to the VP7-177(P₁)-f₁ALDSWKf₂, VP7-177(P₁)-f₃ELDKWAF₄, VP7-177(P₁)-f₅RVLAf₆, VP7-177(P₁)-f₇RILAF₈, VP7-177(P₁)-f₅RVLAf₆f₁ALDSWKf₂ and VP7-177(P₁) proteins were compared. The particulate VP7-HIV proteins were harvested as described in Materials and Methods (5.2.1) and subjected to 12% SDS-polyacrylamide gel electrophoresis (Fig 5.2A). Immunoblot analysis (2.2.7) was then performed on replicas of the SDS-PAGE gels with the HIV-1 subtype C antisera listed in Table 5.1 (Fig 5.2B). The antisera were referred to as BB22, BB14, BB34, BB105, BB81 and IBU3.

The antibodies in HIV-1 subtype C antiserum IBU-3, used in the preliminary experiments, recognized and bound all the above-mentioned gp41 peptides presented by the VP7-HIV proteins, except the subtype B f₃ELDKWAF₄ peptide. Antisera BB22 and BB105 recognised the same HIV peptides as IBU-3. Antisera BB14 and BB81 did not contain antibodies that recognised the subtype C f₁ALDSWKf₂ or subtype B f₃ELDKWAF₄ peptides. The weak or lack of binding of the HIV-1 subtype C antisera to the VP7-177(P₁)-f₁ALDSWKf₂ protein indicated that the subtype C f₁ALDSWKf₂ region may be less immunogenic than the subtype B ELDKWA epitope. Although the quantity of VP7-177(P₁)-f₁ALDSWKf₂ protein on the immunoblots was significantly less than the quantities of the other VP7-HIV fusion proteins, the sensitivity of the immunoblot procedure still allowed the detection of α-f₁ALDSWKf₂ antibodies. The only HIV-1 subtype C antiserum that bound all 5 VP7-HIV proteins, including VP7-177(P₁)-f₃ELDKWAF₄, was BB34. A possible explanation would be that the patient from whom the antiserum was isolated was co-infected with HIV-1 viruses of the B and C subtypes. The subtype C f₅RVLAf₆ and subtype B f₇RILAF₈ peptides were both recognised by all six HIV-1 subtype C antisera, indicating that this region contained a conserved, immunologically important domain. This region was also most likely responsible for the binding of these six antisera to the VP7-177(P₁)-f₅RVLAf₆f₁ALDSWKf₂ protein. No antibody binding to the VP7-177(P₁) vector protein was observed indicating that the signals observed were HIV-specific. These results indicated that certain VP7-HIV proteins, such as VP7-177(P₁)-f₅RVLAf₆ and VP7-177(P₁)-f₇RILAF₈, may be more useful than others in diagnosing HIV-1 subtype C infection while the VP7-177(P₁)-f₃ELDKWAF₄ protein could enable differentiation between the B and C subtypes of HIV. Additional studies with a larger number of HIV-1 subtype C antisera, as well as HIV-1 subtype B antisera, are required to determine the feasibility of such a strategy.

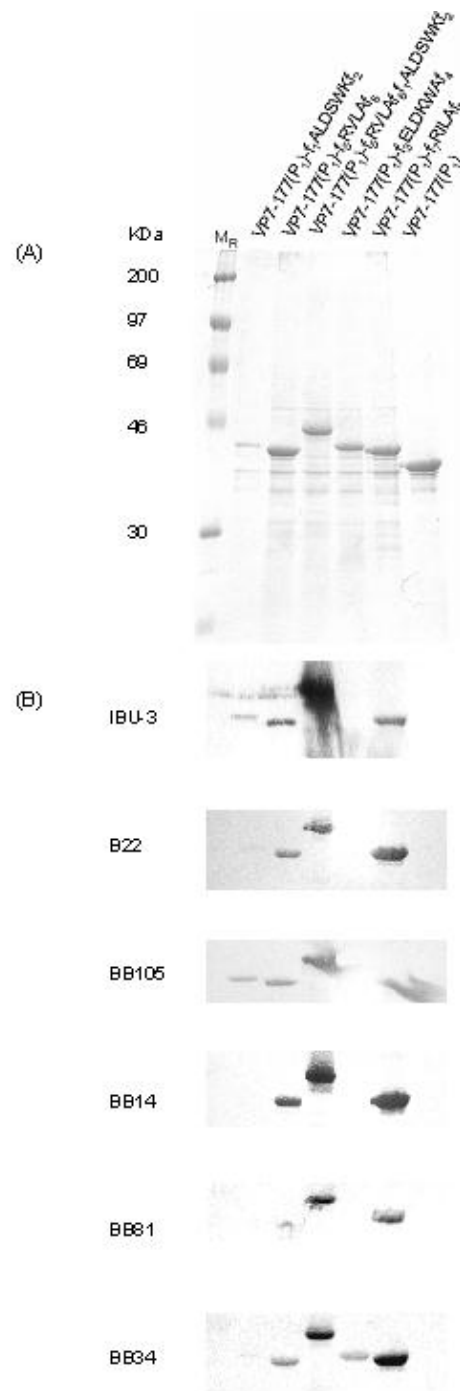


Fig 5.2: SDS-PAGE (A) and immunoblot (B) analysis of particulate proteins VP7-177(P₁)-f₁ALDSWKf₂ (45kDa), VP7-177(P₁)-f₅RVLAf₆ (44kDa), VP7-177(P₁)-f₅RVLAf₆f₁ALDSWKf₂ (50 kDa), VP7-177(P₁)-f₃ELDKWf₄ (45 kDa), VP7-177(P₁)-f₇RILAf₈ (44kDa) and VP7-177(P₁) (39kDa). The protein present in each lane is indicated at the top of the SDS-PAGE gel. M_R is the Rainbow™ coloured protein molecular weight marker (Amersham Pharmacia). The relevant sizes are indicated in kDa. The immunoblots were performed using human HIV-1 subtype C antisera IBU-3, BB22, BB105, BB14, BB81 and BB34 as indicated.

5.3.2 RVLAIERYLKD-specific antibodies in a human HIV-1 subtype C antiserum

All the HIV-1 subtype C antisera investigated in this study contained antibodies that recognised and bound the VP7-177(P₁)-f₅RVLAf₆, VP7-177(P₁)-f₇RILAf₈ and VP7-177(P₁)-f₅RVLAf₆f₁ALDSWKf₂ proteins (5.3.1). It was, however, uncertain if the antibodies were binding to the RVLAIERYLKD/RVLAVERYLKD peptides or to amino acid sequences flanking these peptides (f). To investigate proteins VP7-177(P₁)-f₅RVLAf₆, VP7-144(P₂)-177(P₃)-RVLA-200(P₁) and VP7-144(P₂)-177(P₃)-(RVLA)₄-200(P₁) were harvested as described in Materials and Methods and subjected to 12% SDS-polyacrylamide gel electrophoresis analysis (Fig 5.3). A replica of the SDS-PAGE gel was subsequently subjected to immunoblot analysis (2.2.7) with the human HIV-1 subtype C BB22 antiserum (Fig 5.3). The VP7-144(P₂)-177(P₃)-RVLA-200(P₁) and VP7-144(P₂)-177(P₃)-(RVLA)₄-200(P₁) proteins contained only the subtype C RVLAIERYLKD sequence, either as a single copy or a quadruple repeat respectively, while the VP7-177(P₁)-f₅RVLAf₆ protein contained RVLAIERYLKD with flanking subtype C amino acids.

The HIV-1 subtype C BB22 antiserum contained antibodies that recognized and bound the VP7-177(P₁)-f₅RVLAf₆ protein on the immunoblot membrane, as previously (Fig 5.2). However, no antibody binding was detected to the VP7-144(P₂)-177(P₃)-RVLA-200(P₁) protein or to the VP7-144(P₂)-177(P₃)-(RVLA)₄-200(P₁) protein. This suggested that the antibodies in the HIV-1 subtype C antiserum BB22 recognized a region other than the subtype C RVLAIERYLKD amino acid sequence. Should further evidence be found that the remaining HIV-1 subtype C antisera do not contain any RVLAIERYLKD-specific antibodies, this would confirm that the HIV-1 subtype C sequence RVLAIERYLKD does not induce an immune response that is comparable to that induced by the subtype B RILAVEERYLKD epitope (Chen & Dierich, 1998, Dorn et al., 2000, Shafferman et al., 1991). As the HIV-1 subtype C RVLAIERYLKD amino acids were not responsible for the binding of HIV-1 BB22 antibodies to the VP7-177-f₅RVLAf₆ protein, it was investigated whether the subtype C sequence LGLWGCSGKIIC (aa 574-585), containing amino acids equivalent to the HIV-1 subtype B GCSGKLICTT epitope (aa 590-599) (Cotropia et al., 1996, Viveros et al., 2000) may have been responsible for the observed binding.

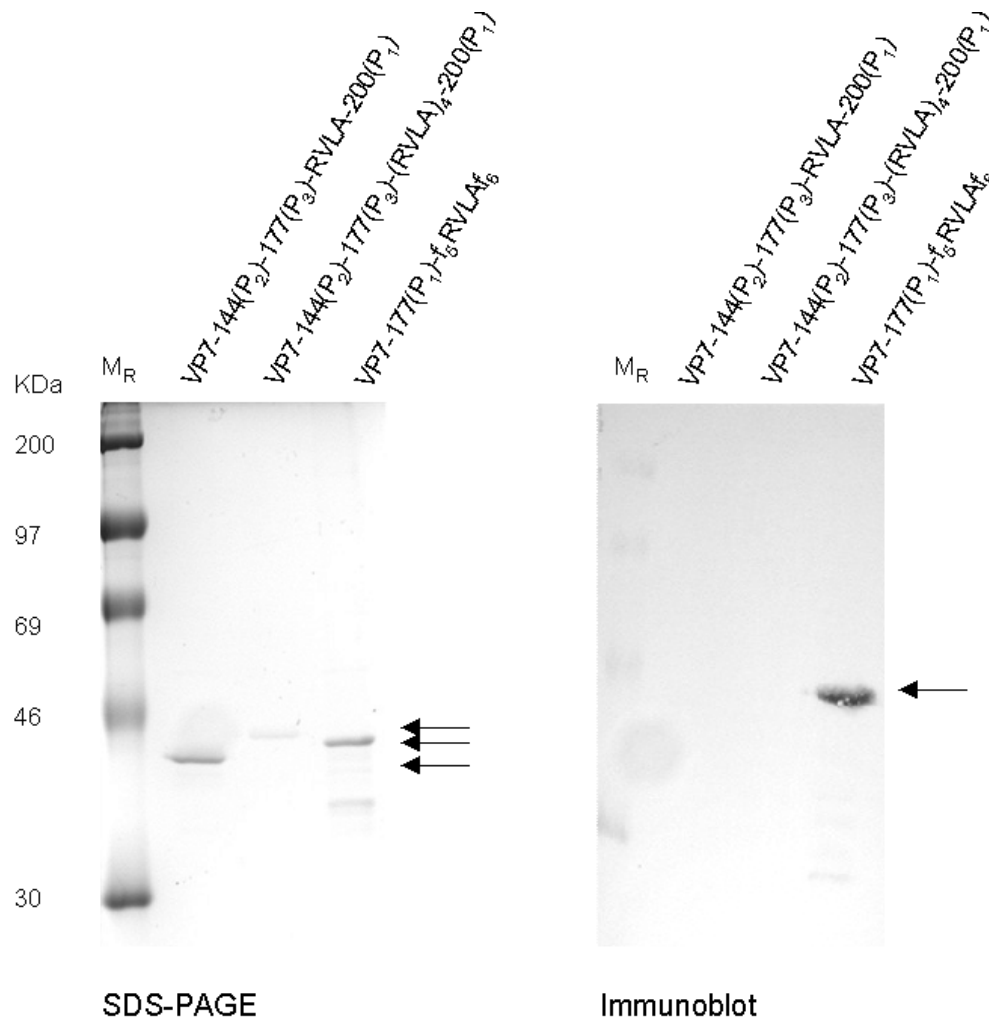


Fig 5.3: SDS-PAGE and immunoblot analysis of the particulate proteins VP7-177(P₁)-f₅RVLAf₆ (44kDa), VP7-144(P₂)-177(P₃)-RVLA-200(P₁) (41kDa) and VP7-144(P₂)-177(P₃)-(RVLA)₄-200(P₁) (46kDa). The protein present in each lane is indicated at the top of the SDS-PAGE gel. The arrow on the side indicates, in each case, the position of the VP7 protein on the gel/membrane. M_R is the Rainbow™ coloured protein molecular weight marker (Amersham Pharmacia). The relevant sizes are indicated in kDa. The immunoblot was performed using human HIV-1 subtype C antiserum BB22.

5.3.3 GCSGKLICTT-specific antibodies in a human HIV-1 subtype C antiserum

The subtype C LGLWGCSGKIIC sequence, containing amino acids equivalent to the subtype B GCSGKLICTT epitope (Cotropia et al., 1996, Viveros et al., 2000), was suggested to be responsible for the binding of antibodies from the HIV-1 subtype C BB22 antiserum to the f₅RVLAf₆ peptide (5.3.2). To investigate the VP7-177(P₁)-f₅RVLAf₆ and VP7-177(P₁)-RCL3R proteins were harvested as described in Materials and Methods and subjected to 12% SDS-polyacrylamide gel electrophoresis analysis (Fig 5.4). A replica of the SDS-PAGE gel was subjected to immunoblot analysis (2.2.7) with the human HIV-1 subtype C BB22 antiserum (Fig 5.4). The VP7-177(P₁)-RCL3R protein contained the subtype C LGLWGCSGKIIC peptide with flanking arginine residues.

Antibodies in the HIV-1 subtype C BB22 antiserum recognized and bound the VP7-177(P₁)-f₅RVLAf₆ protein on the immunoblot membrane, as previously (Fig 5.2 and Fig 5.3). However, no antibody binding to the VP7-177(P₁)-RCL3R protein was detected. This indicates that not only do the antibodies in the HIV-1 subtype C antiserum BB22 not recognize the subtype C RVLAIERYLKD amino acid sequence, they also do not recognize the subtype C LGLWGCSGKIIC sequence, containing amino equivalent to the subtype B GCSGKLICTT epitope (Cotropia et al., 1996, Viveros et al., 2000). Should the remaining HIV-1 subtype C antisera also not contain any subtype C LGLWGCSGKIIC-specific antibodies it could be concluded that this subtype C sequence does not induce an immune response comparable to the equivalent subtype B GCSGKLICTT epitope. These results indicate that it cannot be assumed that the sequence equivalent of an epitope in one HIV-1 subtype is immunogenic in another subtype. The immunogenic region responsible for the binding of the HIV-1 subtype C antisera to the VP7-177(P₁)-f₅RVLAf₆ and VP7-177(P₁)-f₇RILAf₈ proteins may be delineated in future studies.

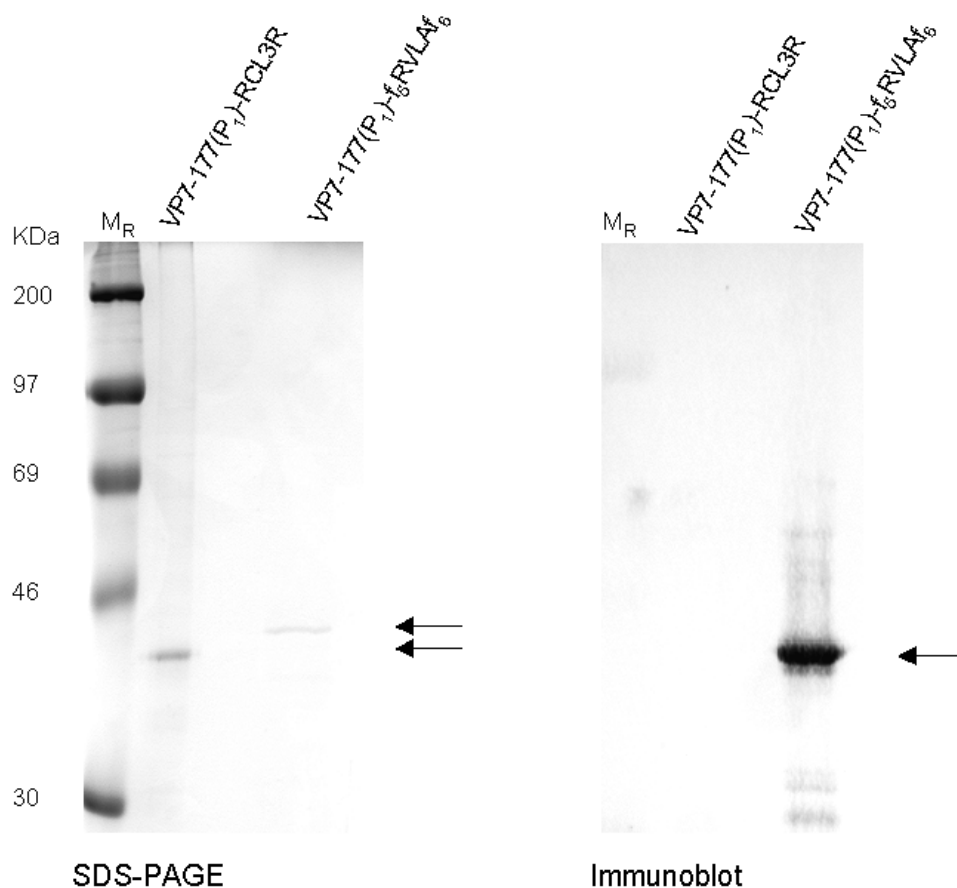


Fig 5.4: SDS-PAGE and immunoblot analysis of the particulate fractions of proteins VP7-177(P₁)-f₅RVLAf₆ (44kDa) and VP7-177(P₁)-RCL3R (41kDa). The protein present in each lane is indicated at the top of the SDS-PAGE gel. The arrow on the side indicates, in each case, the position of the VP7 protein on the gel/membrane. M_R is the Rainbow™ coloured protein molecular weight marker (Amersham Pharmacia). The relevant sizes are indicated in kDa. The immunoblot was performed using human HIV-1 subtype C antiserum BB22.

5.3.4 ELDKWA-specific antibodies in a human HIV-1 antiserum

BB34 was the only HIV-1 subtype C antiserum that contained antibodies that recognized and bound the VP7-177(P₁)-f₃ELDKWaf₄ protein in this study (5.3.1). To determine whether the antibodies in this serum bound the subtype B ELDKWA epitope or the subtype B amino acids flanking this epitope, the VP7-177(P₁)-f₃ELDKWaf₄ and VP7-177(P₁)-ELDKWA proteins were harvested as described in Materials and Methods and subjected to 12% SDS-polyacrylamide gel electrophoresis analysis (result not shown). A replica of the SDS-PAGE gel was subsequently subjected to immunoblot analysis (2.2.7) with the human HIV-1 subtype C BB34 antiserum (Fig 5.5). The VP7-177(P₁)-ELDKWA protein contained only the subtype B epitope.

Antibodies within the HIV-1 subtype C BB34 antiserum recognized and bound the VP7-177(P₁)-f₃ELDKWaf₄ protein on the immunoblot membrane, as previously (Fig 5.2). In addition, this antiserum also bound the VP7-177(P₁)-ELDKWA protein indicating the presence of ELDKWA-specific antibodies. As this antiserum also bound weakly to the VP7-177(P₁)-f₁ALDSWKf₂ protein, it was assumed that the patient may have been co-infected with the HIV-1 B and C subtypes.

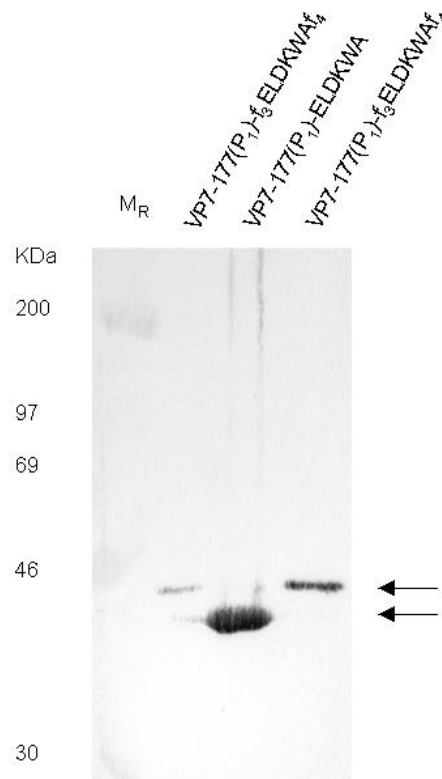


Fig 5.5 SDS-PAGE and immunoblot analysis of the particulate proteins VP7-177(P₁)-f₃ELDKWaf₄ (45kDa) and VP7-177(P₁)-ELDKWA (40kDa). The protein present in each lane is indicated at the top of the SDS-PAGE gel. The arrow on the side indicates, in each case, the position of the VP7 protein on the gel/membrane. M_R is the Rainbow™ coloured protein molecular weight marker (Amersham Pharmacia). The relevant sizes are indicated in kDa. The immunoblot was performed using human HIV-1 subtype C antiserum BB34.

5.4 Discussion

The VP7 fusion proteins with HIV-1 gp41 peptide inserts are largely insoluble and have, therefore, shown very little potential for use in vaccine display strategies. However, these fusions have shown potential in the diagnostic analysis of antiserum from HIV-1 infected patients. This aspect was further explored in this study. It was of interest to determine whether antibodies in HIV-1 patient sera were able to recognise any of the HIV-1 peptides that were inserted into the VP7 top domain. These peptides originated from the immunologically important membrane proximal external region (MPER) of the gp41 protein from HIV-1 subtypes B and C. To investigate, 6 different antisera from HIV-1 subtype C infected patients (Table 5.1) were tested for their ability to bind the VP7-177(P₁)-f₁ALDSWKf₂, VP7-177(P₁)-f₃ELDKWaf₄, VP7-177(P₁)-f₅RVLAf₆, VP7-177(P₁)-f₇RILAf₈, VP7-177(P₁)-f₅RVLAf₆f₁ALDSWKf₂ fusion proteins. Not only did the antisera recognize and bind one or more of the peptides but differences in the binding profiles of these antisera were also observed.

As the VP7 fusion proteins were denatured prior to SDS-electrophoresis, antibodies binding these proteins on the immunoblot membranes were conformation-independent and recognized linear domains on the HIV-1 MPER gp41 peptides. This was not surprising as the subtype B ELDKWA (aa 655-660) and RILAVEERYLKD (aa 572-582) epitopes are conformation-independent, linear epitopes (Chen & Dierich, 1998, Dong et al., 2001, Dorn et al., 2000, Muster et al., 1993) and the subtype C peptides may thus also contain linear epitopes. Although four out of the six subtype C antisera contained antibodies that bound the f₁ALDSWKf₂ peptide, two of the antisera (BB14 and BB81) did not contain any detectable levels of α -f₁ALDSWKf₂ antibodies. Although it has not been shown that antibodies in the subtype C antisera are directed against the subtype C epitope-equivalent peptide ALDSWK, the weak or lack of humeral immune response against the subtype C f₁ALDSWKf₂ peptide indicates that the ALDSWK sequence is less immunogenic than the subtype B ELDKWA epitope (Dong et al., 2001, Muster et al., 1993). None of the antisera, except one, contained α -f₃ELDKWaf₄ antibodies. This indicated that, if binding of the f₁ALDSWKf₂ peptide was due to α -ALDSWK antibodies, these antibodies were subtype C-specific and did not recognize the subtype B ELDKWA sequence. The broadly neutralizing monoclonal 2F5 antibody (Conley et al., 1994, Muster et al., 1994, Muster et al., 1993, Purtscher et al., 1994, Trkola et al., 1995) binds primarily to the core amino acid sequence LDKW of the subtype B ELDKWA epitope (Muster et al., 1994, Muster et al., 1993, Ofek et al., 2004, Purtscher et al., 1996, Purtscher et al., 1994, Sattentau et al., 1995). Should the α -ALDSWK antibody bind the core amino acid sequence LDSW of subtype C, this may explain its inability to recognise the subtype B LDKW core sequence. The presence of α -ELDKWA or α -ALDSWK antibodies may thus be a promising strategy to distinguish between HIV-1 subtype B and subtype C-infected patients, respectively. Further investigation of such a strategy is required and would entail determining the ability of a significant number of subtype B and C antisera to recognise the f₃ELDKWaf₄ and f₁ALDSWKf₂ peptides. The presence of both the α -f₃ELDKWaf₄ and α -f₁ALDSWKf₂ antibodies in antiserum BB34 may be indicative of the patient being co-infected with the B and C subtypes of HIV-1.

All six of the subtype C antisera contained antibodies that bound the f₅RVLAf₆ peptide. Binding to the subtype B f₇RILAf₈ peptide was also observed for each of the six subtype C antisera. This indicated that these antisera may have contained antibodies that were directed against a region within these subtype B and C peptides that was conserved. To determine whether the subtype C RVLAIERYLKD (aa 560-570) sequence, equivalent to the subtype B RILAVEERYLKD (aa 572-582) epitope (Chen & Dierich, 1998, Dong et al., 2001, Dorn et al., 2000), was responsible for the subtype C antisera recognition of the subtype C f₅RVLAf₆ peptide, this sequence was presented as a single copy, or as a quadruple repeat, without any flanking amino acids by the VP7-144(P₂)-177(P₃)-200(P₁) vector protein (Van Rensburg, 2004). We found that the subtype C epitope-equivalent sequence RVLAIERYLKD (aa 560-570) was not responsible for the BB22 antiserum binding the f₅RVLAf₆ peptide. If the remaining HIV-1 subtype C antisera do not contain any RVLAIERYLKD-specific antibodies, it can be concluded that the subtype C sequence RVLAIERYLKD is not as immunogenic as the subtype B RILAVEERYLKD epitope (Chen & Dierich, 1998, Dorn et al., 2000, Shafferman et al., 1991). Antibodies from the HIV-1 BB22 antiserum thus recognized and bound sequences located in the RVLAIERYLKD flanking domains. The subtype C sequence LGLWGCSGKIIC (aa 574-585), containing amino acids equivalent to the HIV-1 subtype B GCSGKLICTT epitope (aa 590-599) (Cotropia et al., 1996, Viveros et al., 2000), was present within the RVLAIERYLKD flanking domain f₆. The LGLWGCSGKIIC amino acid sequence was, however, not responsible for BB22 antiserum binding to the subtype C f₅RVLAf₆ peptide. Future studies may delineate the immunogenic region within the f₅ and/or f₆ domains responsible for the antibody binding observed.

Antibodies directed against the ELDKWA sequence were detected within the BB34 serum and confirmed the immunogenicity of this subtype B epitope (Dong et al., 2001, Muster et al., 1993). As we have shown that the subtype C ALDSWK and RVLAIERYLKD sequences are less immunogenic than their equivalent subtype B ELDKWA and RILAVEERYLKD epitopes, it is concluded that the immunogenicity of sequences in one subtype cannot necessarily be inferred from the immunogenicity of sequences in another subtype.

A recent study has identified additional sequences within the subtype B ELDKWA and RILAVEERYLKD flanking domains that may be recognised by antibodies from the antisera in this study. Sequences WLWYIKIFI (aa671-679) and YIKIFIMII (aa 674-682), identified as putatively immunogenic (Gupta et al., 2011), are both present in the f₃ELDKWaf₄ peptide and, in addition to the ELDKWA epitope, may be responsible for the recognition of this peptide by the BB34 antiserum. Equivalent to these sequences are the subtype C sequences WLWYIKIFI (aa659-667) and YIKIFIMIV (aa662-670) present in the f₁ALDSWKf₂ peptide, but these sequences are most likely to be less immunogenic judging by the weak immune response elicited by subtype C infected individuals against the subtype C f₁ALDSWKf₂ peptide. The same study also identified a putative epitope, QLQARVLAV (aa568-576) (Gupta et al., 2011) present within the f₇RILAf₈ peptide. This sequence, as well as equivalent subtype C sequence QLQTRVLAI (aa556-564), may be sufficiently conserved to be responsible for the binding of the six subtype C antisera to the subtype B f₇RILAf₈ and subtype C f₅RVLAf₆ peptides in this study.

The application of VP7 fusion proteins in antiserum characterization and disease diagnosis may be expanded to other infectious agents and is dependent on the pathogen-specific peptides presented by the VP7 protein. The diagnosis of FMD in infected animals, for example, has recently been explored using VP7 protein presenting the FMD VP1 immunodominant domain (aa 129-164) (Results not shown).

Chapter 6

Concluding remarks

Bluetongue virus (BTV) major capsid protein 7 (VP7) trimers, on the surface of BTV core-like particles (CLPs), had previously been used to present foreign peptides to the immune system (Adler et al., 1998, Belyaev & Roy, 1992, Le Blois & Roy, 1993, Roy, 1996a, Roy & Sutton, 1998). Although peptides up to 39 amino acids in length have reportedly been inserted into the VP7 protein without affecting CLP stability (Roy et al., 1997), it is likely that the insertion of longer peptides may interfere with the formation of stable particles, as reported elsewhere (Janssens et al., 2010, Peralta et al., 2009, Tanaka et al., 1995, Tissot et al., 2010). When expressed in *Spodoptera frugiperda* (Sf9) insect cells by means of a recombinant baculovirus strategy, the VP7 trimers of African horsesickness virus (AHSV), a closely related orbivirus, have been observed to aggregate and assemble into unique crystal-like particles (Chuma et al., 1992, Maree et al., 1998, Monastyrskaya et al., 1997). As these particles were immunogenic (Wade-Evans et al., 1997), they were proposed as a means of displaying foreign antigenic peptides to the immune system and the top domain of the AHSV-9 VP7 protein was subsequently modified to facilitate the insertion of foreign peptides (Maree, 2000, Riley, 2003, Van Rensburg, 2004). Although the insoluble crystal-like VP7 particles were initially suggested as a novel particulate antigen display system, in this study it was found that soluble chimeric VP7 trimer molecules, rather than VP7 trimers assembled into insoluble particles, present the best display strategy for eliciting a strong insert-specific humoral immune response.

The insertion of 6-mer peptides downstream of amino acids 144, 177 or 200 within the top domain of VP7 (aa121-249) during vector construction affected the solubility of the intrinsically insoluble AHSV-9 VP7 protein. The insertion of larger foreign peptides, from 11 to 239 amino acids in length, also affected VP7 solubility. It was determined that both the VP7 top domain insertion site as well as the nature of the inserted peptide influence the overall equilibrium between soluble chimeric VP7 trimers and VP7 particles. It is, however, not yet possible to predict chimeric VP7 protein solubility and the solubility of each construct needs to be individually assessed. It was also found that, unlike VP7 protein solubility, the ability of the chimeric VP7 proteins to form trimers was not affected. The only exception was the VP7-200(P₁)-eGFP protein, the majority of which occurred in a monomeric rather than in a trimer form. As the insertion of the 239 amino acid enhanced green fluorescent protein (eGFP) downstream of either amino acid 177 or 144 did not interfere with trimer formation, this indicated that the AHSV VP7 trimer is capable of accommodating even large insertions within its top domain without losing its ability to form trimers. Although the WT VP7 trimer molecule has been shown to be stable even under strong denaturing conditions, such as in the presence of SDS at room temperature, the stability of the chimeric VP7 trimers under these conditions was dependent mainly on the site of insertion. Trimers with peptides inserted downstream of

VP7 amino acid 177 were found to be the most stable while trimers with peptide insertions downstream of amino acid 200 were the least stable. The inserted peptide was also found to play a role in trimer stability in some cases.

As the insertion of a peptide (aa129-164) from the outer capsid protein VP1 of foot-and-mouth disease virus (FMDV) into site 177(P₁) yielded both an insoluble particulate VP7-177(P₁)-FMDVepi protein fraction as well as a significant soluble protein fraction (40%), a comparison of the FMDV-specific, neutralizing humeral immune response elicited by these two fractions in guinea pigs indicated, surprisingly, that it was the soluble VP7-177(P₁)-FMDVepi trimers that elicited the stronger humeral immune response. This result not only indicated that a foreign peptide inserted into the VP7 top domain is presented on the surface of VP7 trimers in its native immunogenic state, but also that soluble chimeric VP7 trimers elicit a stronger humeral immune response than trimers aggregated into insoluble particles. This is most likely due to the increased number of copies of the foreign peptide being presented to the immune system on the surface of non-aggregated, soluble trimers. Thus our study focused instead on the use of soluble chimeric VP7 trimers as an antigen display system. Soluble VP7 monomers were not considered as a display system due to their scarcity and low yields under normal conditions. Although 6.5mg of soluble VP7-177(P₁)-FMDVepi trimers could be harvested from 1×10^8 recombinant baculovirus-infected insect cells, various strategies to increase the soluble trimer fraction as well as the solubility of other less soluble chimeric VP7 proteins were subsequently investigated. The substitution of an uncharged leucine residue in position 345 within the bottom domain of AHSV-9 VP7 with a positively charged arginine residue (Meyer, 2002) significantly increased the solubility of the modified proteins and did not affect their ability to form trimers. This modification may be incorporated into future VP7 vector proteins to increase protein solubility. Treatments with amino acid L-arginine and the ionic detergent sarkosyl were also found to be efficient at reversing VP7 trimer-trimer aggregation and increasing VP7 protein solubility. L-arginine reversed loose aggregation of correctly folded VP7 trimer molecules while sarkosyl solubilized both correctly folded and incorrectly folded trimer aggregates. Inoculation of guinea pigs with chimeric VP7 antigen in the presence of L-arginine or sarkosyl elicited a more consistent, strong immune response than untreated soluble VP7-177(P₁)-FMDVepi trimers. This indicated that these treatments did not negatively affect the immunogenicity of the antigen and their presence ensured that the antigen is presented in a soluble, non-aggregated form to the immune system. Although the 3 log₁₀ FMDV neutralization titers, observed in guinea pigs immunized with VP7-177(P₁)-FMDVepi trimers in the presence of L-arginine or sarkosyl, was much higher than the 2 log₁₀ neutralization titers that are normally considered sufficient to protect cattle against FMDV infection (Barnett et al., 2003), these results cannot be extrapolated to larger host animals (Alves et al., 2009, Kamstrup et al., 2006) and similar immunogenicity trials in cattle are currently underway. Although equivalent humeral immune responses were elicited by L-arginine-treated and sarkosyl-treated VP7-177(P₁)-FMDVepi trimers, unlike sarkosyl, L-arginine solubilizes only correctly folded trimer aggregates and is not required to be removed from the immunogen solution prior to inoculation. These advantages and the observation that VP7 fusion proteins may be

freeze-dried in the presence of L-arginine and remain fully soluble following reconstitution in water, have led to L-arginine being the preferred solubilization agent in our laboratory.

The eGFP protein was found to fold equally well into its functional, fluorescing native conformation when inserted into any of the three VP7 top domain insertion sites and presented on the surface of VP7 trimers. It has, however been observed that correctly folded VP7-144 fusion trimers, such as VP7-144(P₂)-eGFP and VP7-144(P₂)-VP2(110aa), tend to form loose trimer-trimer aggregates that can be readily solubilized by simple dilution or treatment with L-arginine. This tendency has formed the basis of a low speed centrifugation strategy designed to harvest large quantities of soluble, correctly folded VP7 fusion trimers.

The small animal trials conducted in this study also gave an indication as to which adjuvant should be administered with the antigen in order to elicit the most effective antigen-specific immune response. Incomplete Freund's adjuvant (IFA), lacking the *Mycobacterium tuberculosis* component (Oscherwitz et al., 2006), was found to be as effective at stimulating the immune response against soluble VP7-177(P₁)-FMDVepi trimers in guinea pigs as Complete Freund's adjuvant. IFA may thus be used as an alternative to CFA in future animal studies in order to minimize the adverse localized reaction of animals to the *Mycobacterium tuberculosis* component. Montanide™ ISA 206 (Barnett et al., 1996) may also be used as a safer alternative to CFA as we found that this adjuvant was as effective at eliciting the humeral immune response against L-Arginine-solubilized VP7-177(P₁)-FMDVepi trimers in guinea pigs as the CFA adjuvant. The commonly used aluminium hydroxide (Al(OH)₃)/Saponin adjuvant (Marrack et al., 2009) was, however, not as effective at eliciting an immune response against L-arginine-solubilized VP7-177(P₁)-FMDVepi trimers as the Montanide™ and/or CFA adjuvants, and thus will no longer be considered for use in future animal trials.

When expressed in recombinant baculovirus-infected insect cells, the VP7-HIV fusion protein in this study were found to be largely insoluble and were explored with respect to their potential to detect HIV antibodies in HIV patient sera. Not only did the VP7-HIV fusion proteins on an immune blot bind non-conformational, insert-specific antibodies from the HIV patient antisera but different binding profiles were observed that could be used to characterize different HIV-1 subtype C antisera or used to distinguish between subtype B and C infected individuals. This strategy needs to be further explored with a larger number of antisera from both subtype B and subtype C-infected patients. One of the advantages of this strategy is that large quantities of protein may be expressed and harvested using the baculovirus expression system which would be useful in screening large numbers of antisera. This strategy may also be expanded to the characterization of antisera from patients infected with other pathogens and would depend on the availability of VP7 constructs with pathogen-specific peptide sequences.

The results in this study indicate that foreign peptides may be displayed by the AHSV major capsid protein VP7 either on the surface of soluble VP7 trimers as a vaccine display system or by insoluble particles, in a denatured

form on immunoblots, as an antiserum characterization strategy. The display of antigenic peptides in a soluble form on the surface of VP7 trimers is especially important in cases where expression in a heterologous expression system results in a peptide/protein being insoluble. The neutralization-specific, outer capsid protein VP2 has been the target of most recombinant vaccine strategies against AHSV (Guthrie et al., 2009, Roy, 1996b). However, this protein is insoluble when expressed in insect cells (Scanlen et al., 2002). In addition, shorter AHSV VP2-specific peptides expressed in bacterial or insect cells are also insoluble and non-immunogenic (Venter et al., 2000). The ability of soluble VP7-177(P₁)-VP2epiB and VP7-144(P₂)-VP2(110aa) trimers to elicit a insert-specific humeral immune response in this study indicated that the VP7 trimers can be used to present VP2 peptides to the immune system in a soluble, immunogenic form and may eventually lead to the development of a recombinant AHSV vaccine.

Modification of the AHSV VP7 has also been shown to increase this proteins solubility and thus co-expression of a soluble, chimeric VP7 protein with the WT AHSV VP3 protein in recombinant baculovirus-infected insect cells may lead to an increased yield of chimeric core-like particles (CLPs). The insolubility of the AHSV VP7 protein has previously been thought to be responsible for the low yield of AHSV core-like particles (CLPs) observed in infected insect cells (Maree et al., 1998). Although the size of the presented peptide may be limited by a CLP strategy (Janssens et al., 2010, Peralta et al., 2009, Tanaka et al., 1995, Tissot et al., 2010), the larger CLP particles may be capable of inducing cellular immunity. Although not yet explored, it is likely that the soluble VP7 trimers may only elicit a humeral immune response and not a cellular immune response due to their small size. This strategy will be investigated shortly. Another avenue that is currently being explored is the use of the soluble, fluorescing VP7-eGFP protein trimers to investigate VP7 transport within the cell under the electron microscope as well as trimer-trimer interactions.

ABBREVIATIONS

aa	amino acid
Ab	antibody
AcNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
AHS	African horsesickness
AHSV	African horsesickness virus
AIDS	Acquired immunodeficiency syndrome
Al(OH) ₃	Aluminium hydroxide
amp	ampicillin
AP	Ammonium persulphate
APC	Antigen presenting cells
Arg	L-Arginine
ATP	Adenosine triphosphate
β-gal	Beta-galactosidase
β-ME	Beta-mercaptoethanol
bp	base pairs
BSA	bovine serum albumin
BT	Bluetongue
BTV	Bluetongue virus
CaCl ₂	calcium chloride
CO ₂	Carbon dioxide
°C	Celsius degree
CFA	Complete Freund's adjuvant
CLP	Core-like particle
Cryo-EM	Cryoelectron microscopy
CTL	Cytotoxic T lymphocyte response

dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dUTP	2'-deoxyuracil-5'-triphosphate
dH ₂ O	distilled water
ddH ₂ O	double distilled water
DIVA	Differentiation between infected and vaccinated animals
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	2'-deoxy-nucleotide-5'-triphosphate
ds	double stranded
dsDNA	double stranded deoxyribonucleic acid
DTT	1,4-dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetra-acetic acid
e.g.	exempli gratia (for example)
eGFP	enhanced Green Fluorescent Protein
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope
Epi	Epitope
et al.	et alia (and others)
EtBr	ethidium bromide
EtOH	ethanol
FB-RS2	established pig kidney cell line
FCS	foetal calf serum

Fig.	Figure
FMD	Foot and mouth disease
FMDV	Foot and mouth disease virus
FU	Fluorescence units
GFP	Green Fluorescent protein
GP	Guinea pig
gp41	transmembrane glycoprotein 41
gp120	surface glycoprotein 120
gp160	glycoprotein 160
GST	Glutathione S-transferase
HCl	hydrochloric acid
HIV	Human immunodeficiency virus
H ₂ O	water
hr	hour
IFA	Incomplete Freund's adjuvant
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
KDa	KiloDalton
λII	lambda DNA molecular weight marker II
LA	Live attenuated virus
LB broth	Luria-Bertani broth
LCMV	Lymphocytic choriomeningitis virus
M	molar
mA	milliampere
mAb	monoclonal antibody
MCS	multiple cloning site
mg	milligram

MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
MHC	Major Histocompatibility Complex
μg	microgram
μl	microliter
μm	micrometer
min	minute
ml	millilitre
mM	millimolar
m.o.i.	multiplicity of infection
Montanide	Montanide™ ISA 206 polymeric gel adjuvant
M _p	PageRuler™ prestained protein ladder
M _R	Rainbow™ coloured protein molecular weight marker
Mr	molecular weight
mRNA	messenger ribonucleic acid
m/v	mass per volume
MVA	Modified vaccinia Ankara
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
NCID	National Centre for Infectious Diseases
ND	Nondenatured
ng	nanogram
NIV	National Institute of Virology
nm	nanometer
NP-40	Nonidet® -P40
NS	Nonstructural protein
OBP	Onderstepoort Biological Products

OD	optical density
OIE	Office international des Epizooties
O/N	overnight
OVI	Onderstepoort Veterinary Institute
ϕ X	PhiX174/ <i>Hae</i> marker
PAGE	polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
p.i	post infection
pmol	picomol
pol	polymerase
PSB	protein solvent buffer
R.E.	Restriction Endonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SA	South Africa
Sarkosyl	Na ⁺ <i>N</i> -lauroylsarcosine
SAT	South African type
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second
SEM	Scanning electron microscopy
Sf9	<i>Spodoptera frugiperda</i> insect cells
SIV	Simian immunodeficiency virus

ss	single stranded
STE	Sodium chloride-Tris-EDTA buffer
TADP	Transboundary Animal Diseases Programme
TAE	Tris-acetate-EDTA
<i>Taq</i>	<i>thermus aquaticus</i>
TCID ₅₀	Median tissue culture infective dose
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
tet	tetracycline hydrochloride
TGS	Tris-Glycine-Sodium dodecyl sulphate buffer
Th	T helper cell
Tris	Tris-(hydroxymethyl)-aminomethane
U	units
USA	United States of America
UV	ultraviolet
V	volts
VLP	Virus-like particle
VN	Virus neutralization
VP	viral protein
v/v	volume per volume ratio
WHO	World Health Organisation
wt	wild type
w/v	weight per volume ratio
X-gal	5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside
ZIM	Zimbabwe

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