CHAPTER 3:

IMMUNOLOGICAL DISPLAY OF THE N-TERMINAL AMINO ACIDS OF NS3

3.1 INTRODUCTION

AHSV NS3 and NS3A are encoded from the same open reading frame and are identical except for an additional 10 or 11 amino acids (aa), depending on the serotype, at the N-terminus of NS3 (Van Staden *et al.,* 1995). Polyclonal antibodies (anti-β-gal-NS3 and anti-NS3) directed against either prokaryotic or eukaryotic expressed NS3, therefore, detect both NS3 and NS3A in AHSV infected cells. Localisation studies using these antiserums indicated that NS3 and/or NS3A are associated with the membranes at sites of viral release (Stoltz *et al.,* 1994). The NS3 and NS3A proteins may therefore interact with the viral particle and facilitate release from infected cells. As the NS3 and NS3A proteins could not be distinguished it was unclear whether both proteins display a similar localisation pattern and if both are involved with and necessary for virus release.

The availability of antibodies that are specifically directed against the N-terminal extension of NS3 may potentially be of great benefit in distinguishing between NS3 and NS3A. Antiserum directed against this region of NS3, that is absent in NS3A, would specifically detect NS3 and could be used in localisation studies to identify the regions of the cell specifically associated with NS3 during AHSV infection. This could be compared to localisation studies using the α-β-gal-NS3 serum, and the localisation of NS3 and NS3A determined, should they differ. In this way NS3 and NS3A could be distinguished.

In order to address the problem of preparing antibodies against such small peptide region we explored the possibility of using AHSV VP7 as a delivery system for presenting small peptides to the immune system. AHSV VP7 is a highly hydrophobic protein of 349 amino acids (Roy *et al.,* 1991) and a group-specific immunogenic antigen (Wade-Evans *et al.,* 1997). In AHSV infected cells (Burroughs *et al.,* 1994) or recombinant baculovirus infected cells (Chuma *et al.,* 1992) VP7 aggregates as large, flat hexagonal crystals that appear to be unique to AHSV. These disc-shaped crystals are visible under the light microscope and vary both in number per cell (usually between 1 and 3) and size. The

crystals consist of VP7 trimer subunits that aggregate via interactions between hydrophobic residues to form flat hexagonal sheets (Basak *et al.*, 1996). Each VP7 monomer is composed of two distinct domains. The top (aa 121-249) domain is folded into an anti-parallel β-sandwich while the lower domain (aa 1-120 and 250-348) is composed of α-helices with extended loops between them (Grimes *et al.*, 1995).

The crystallographic structure of VP7 was used to identify hydrophilic regions on the surface of the protein that may be accessible to the environment. Unique restriction enzyme sites for cloning purposes have been inserted into these exposed regions at residues 144-145, 177-178 and 200-201 of AHSV-9 VP7 (Maree, 2000). The resulting insertion mutants were designated VP7mt144, VP7mt177 and VP7mt200 respectively. VP7mt200 has *Hin*dIII, *Xba*I and *Sal*I sites at position 200. This mutated VP7 gene has been cloned into the transfer vector pFASTBAC1 and the protein expressed as a baculovirus recombinant. The insertion was found not to alter the ability of VP7 to form highly ordered crystals (Maree, 2000). This VP7 insertion mutant therefore displays a highly ordered predictable structure, is immunogenic and has a unique cloning site and should therefore fulfil the requirements for an antigen display vector.

It was decided to use the VP7mt200 vector for the display of the N-terminal amino acids of NS3. In this chapter the cloning of the DNA sequence encoding the first 12 amino acids of AHSV-3 NS3 into the cloning site at position 200 of VP7mt200 is described. The resulting chimera, VP7-NS3, was expressed as a baculovirus recombinant and compared to the native VP7 and VP7mt200 proteins in terms of its physical and morphological properties. The VP7-NS3 chimera was subsequently purified and used for the production of polyclonal antiserum directed against NS3.

3.2 MATERIALS AND METHODS:

3.2.1 Cloning 12 N-terminal amino acids of NS3 into VP7mt200

For the cloning of the N-terminal amino acids of NS3 into the vector VP7mt200, two complementary synthetic 53-mer oligonucleotides, with sequences: 5'GCTCTAGAATGAGTCTAGCTACGATCGCCGAAAATTATATGATG***GTCGAC***GAC3' and 5'GTC***GTCGAC***CATCATATAATTTTCGGCGATCGTAGCTAGACTCATTCTAGAGC3' were synthesized. The primers were identical to nucleotides 20 - 55 of the coding strand of the NS3 gene of AHSV-3 (Van Staden, 1993) or its complementary region on the noncoding

strand. This represents the region encoding the first 12 amino acids of NS3. The NS3 sequences were flanked by *Xba*I (underlined) and *Sal*I (bold italics) sites and two or three additional nucleotides to stabilise their 5' ends. A 60 µl mixture was prepared containing 100 pmol of each oligonucleotide in TE buffer. The mixture of the two primers was heated to 95°C for 5 min and placed at 65°C for 1 h. The primer mixture was gradually cooled to room temperature and placed on ice before use. The annealed double stranded oligonucleotide was then digested with *Xba*I and *Sal*I in the appropriate buffer at 37°C for 4 h. The digested product was precipitated in 96% ethanol for 1 h on ice and collected by desktop centrifugation for 15-30 min. Residual salt was removed by washing the pellet with 70% ethanol and recentrifuging. The insert was then dried, resuspended in UHQ and analysed electrophoretically on a 2% agarose gel.

The plasmid pFB-7mt200 was obtained from FF Maree (University of Pretoria) and represents the VP7 insertion mutant, VP7mt200, containing *Hin*dIII, *Xba*I and *Sal*I sites inserted between residues 200 and 201, cloned into the baculovirus transfer vector pFastBac1. The plasmid was digested first with *Sal*I for 1 h and then with *Xba*I for 3 h in the same reaction mixture. The linearised pFB-7mt200 and the NS3-specific insert were ligated and transformed into *E. coli* cells. Plasmid DNA was extracted from a number of possible recombinant colonies and analysed by restriction enzyme digestion and nucleotide sequencing (protocols as described in 2.2.4.6 and 2.2.5). Recombinants were designated pFB-VP7-NS3.

3.2.2 Baculovirus expression of VP7-NS3

Recombinant baculoviruses expressing the VP7-NS3 chimera, AHSV-9 VP7 and VP7mt200 were prepared as described in section 2.2.6. Expression of VP7-NS3 was analysed on 12% SDS-PAGE gels. Gels were stained in 0.125% Coomassie Blue, 50% methanol, 10% acetic acid and destained in 5% acetic acid, 5% methanol. Baculovirus expressed VP7, VP7mt200 and VP7-NS3 were analysed by Western blot (2.2.2.4) with anti-AHSV9, anti-NS3 and anti-β-gal-NS3 sera as primary antisera.

3.2.2.1 Plaque purification

Pure recombinant viral stocks were prepared from single plaques using standard virological methods (O'Reilly *et al.*,1992). Briefly, *Sf9* cells were seeded in 6-well plates as described under 2.2.6.4. Dilution series of the VP7, VP7mt200 and VP7-NS3 recombinant baculovirus were prepared, from 1 x 10^{-1} to1 x 10^{-6}, in 1 ml medium. The medium from each well was removed and replaced with the virus dilution. The plates were incubated at room temperature for 2 h, after which the inoculum was replaced with sterile 3% low melting agarose at 37°C diluted in an equal volume Grace's medium. The infected cells were incubated at 27°C for 4 days and then stained overnight with 1 ml Neutral Red (100 µg/ml in Grace's medium). Baculovirus plaques were plucked as agarose plugs with a sterile Pasteur pipette, placed in 1 ml Grace's medium and resuspended by vortexing. Virus plaques were amplified as described in 2.2.6.5.

3.2.3 Hydrophobicity analysis

Hydropathy plots of AHSV-9 VP7, VP7mt200 and VP7-NS3 were prepared using the Hopp and Woods predictive model (Hopp & Woods, 1981) in the ANTHEPROT package (Geourjon *et al.*, 1991).

3.2.4 Solubility analysis and purification of VP7-NS3

Monolayers of *Sf9* cells were infected with recombinant baculoviruses expressing VP7, VP7mt200 and VP7-NS3 at a MOI of 5-10 pfu/cell and incubated at 27°C. Protein expression was verified under the light microscope after 2 days, and the infected cells harvested by centrifugation at 3000 rpm for 5 min 72 h.p.i. The resulting cell pellet was washed once in 1xPBS, resuspended in 500 µl Lysis buffer (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.25% Nonidet P-40) and incubated on ice for 30 min. 10 strokes of a dounce homogeniser was used to further disrupt the cells, and the nuclei and cell debris removed by centrifugation at 2000 rpm for 5 min. This was repeated three times in order to obtain the maximum amount of crude protein. The cytoplasmic extracts were pooled and separated into soluble and particulate fractions by centrifugation at 5000 rpm for 30 min at 4°C. The fractions were analysed by 12% SDS-PAGE. For purification of the chimeric protein the fraction containing VP7-NS3 was sedimented on a 40 - 75% (w/v) discontinuous sucrose gradient in 0.2M Tris-HCl, pH7.5. The gradient was centrifuged at 12000 rpm for 1 hour at 4°C in a SW41.1 Beckman rotor. Ten 500 µl fractions were collected and a 1/100 sample of each fraction analysed by SDS-PAGE. Fractions containing VP7-NS3 were pooled, diluted 3x in 1xPBS and collected by centrifugation at 22000 rpm for 40 min. Purified VP7-NS3 was analysed by 12% SDS-PAGE and the concentration and purity of the sample determined.

3.2.5 Scanning electron microscopical analysis

Purified VP7-NS3 was fixed in 2.5% Glutaraldehyde in 0.075M KH_2PO_4/Na_2HPO_4 (Na-K-P buffer), pH 7.4 for 30 min. The protein was then filtered onto a 0.2 µm nylon filter, washed three times in Na-K-P buffer and dehydrated by successive treatment in 50%, 70%, 90% and 3x 100% ethanol for 15 min each. The filter was then air dried overnight and mounted onto a stub, spatter coated with gold-beladium and overlayed with a thin layer of carbon. The protein was viewed in a JEOL scanning electron microscope at 5.0 kV.

3.2.6 Immunisation schedule

VP7-NS3 purified as described under 3.2.4 was used to immunise a New Zealand White rabbit. Amounts of antigen were estimated by serial dilution and comparison to protein of known concentration. The immunisation schedule is outlined in Table 3.1, half the antigen was emulsified in ISA 50 (incomplete seppic adjuvant) and half in Quillaja saponin. Both emulsions were then injected intramuscularly into the same rabbit. The rabbit was bled from the ear prior to inoculation and 7 days after the final boost injection. The blood was allowed to clot at 37°C for 1 h and at 4°C overnight. Blood samples were

then centrifuged at 10 000 rpm for 10 min at 4°C and the serum stored at -20°C. Serum was designated anti-SfVP7-NS3.

Table 3.1: Immunisation schedule:

Antigen	Day of immunisation	Amount of antigen (µg)	Adjuvant	Serum obtained
Baculovirus expressed VP7-NS3	1	100	ISA 50 and Quillaja saponin	pre-innoculation serum
	41	70		----------
	70	50		anti-SfVP7-NS3

3.2.7 Evaluation of antiserum raised against VP7-NS3

The antiserum was analysed using two different strategies. The ability of the anti-SfVP7-NS3 to bind NS3 but not NS3A in their denatured forms was tested in a Western blot. The ability to bind NS3 under less denaturing conditions was investigated by immune-precipitation.

3.2.7.1 Western Blot

Sf9 cells seeded at 1x 10^6 cells/well in 6-well plates were infected with wild type baculovirus and recombinant baculoviruses expressing NS3, NS3A and VP7-NS3 at MOI of 5-10 pfu/cell. Cells were harvested as described in 2.2.6.7. Crude protein samples were prepared, separated by SDS-PAGE and immobilised on membranes. A 1/100 dilution of the pre-inoculation serum or the anti-SfVP7-NS3 serum were prepared in 1% blocking solution and used as the primary antibody. Western blots were performed as described in 2.2.2.4.

3.2.7.2 Immune precipitation of NS3:

3.2.7.2.1 *In vitro* transcription and translation of NS3 serotype 3

The plasmid pBS-NS3 containing the NS3 gene from AHSV serotype 3 in the T7 orientation was obtained from C. Smit (Dept. Genetics, University of Pretoria). The plasmid was linearised with *Hind*III, which cleaves in the multiple cloning site (MCS) on the 3' side of the NS3 gene. The digested product was cleaned using the phenol/chloroform-ethanol precipitation protocol described under 2.2.4.3. Digestion was verified by agarose gel electrophoresis.

A 20 µl transcription reaction was prepared containing 15 µl linearised template, 1 µl RNase inhibitor, 100 mM DTT, 2.5 mM of each rNTP, 1 µl T7 RNA polymerase and 2 µl 10 X transcription buffer (PROMEGA). The mixture was incubated for 60 min at 37°C. Transcription efficiency was evaluated on a 0.8% RNA agarose gel.

The translation reactions were carried out using a commercial rabbit reticulocyte lysate kit (Amersham Life Sciences) according to the manufacturer's instructions. The translation mixture contained 2 µl 12.5 x translation mix (without methionine), 1 µl 2.5 M potassium acetate, 0.5 µl 25 mM magnesium acetate, 1.5 µCi/µl [^{35}S] methionine, 10 µl rabbit reticulocyte lysate, approximately 0.5 – 0.8 µg RNA and RNase-free UHQ to a final volume of 25 µl. The reaction was incubated at 30°C for 60-90 min. Protein expression was analysed by SDS_PAGE and autoradiography.

3.2.7.2.2 Immune precipitation

The ^{35}S labeled translation products were incubated in the presence of 5 µl anti-SfVP7-NS3 serum or anti-β-gal-NS3 (positive control) with 0.01M STE for 60 min with shaking at room temperature. Samples were also incubated in the absence of serum as a negative control. Protein A slurry (10% w/v Protein A in 0.01M STE) was added and the mixture incubated as before for 60 min. Bound proteins were collected by desktop centrifugation for 30 sec and the supernatant retained. The pellet was then washed three times in 0.01M STE and resuspended in PBS. Precipitated and unprecipitated proteins were analysed by SDS-PAGE and autoradiography.

3.2.8 Immunogold labeling of purified VP7-NS3

Immunogold labeling experiments were based on the methods of Portner & Murti (1986) and Murti *et al.*(1988). VP7mt200 and VP7-NS3 crystals were purified as described under section 3.2.4. The crystals were adsorbed onto copper 400-mesh Formvar carbon-coated grids and the grids rinsed with TBS (500 mM NaCl and 25 mM Tris, pH 7.6). The grids were then floated on a solution of primary antibody (anti-β-gal-NS3 serum or no serum diluted 25-fold in TBS containing 1% gelatin) for 1 h. Alternatively the purified crystals were incubated overnight at 4°C in the presence of the primary antiserum solution and then adsorbed to the grids. Following thorough rinsing with TBS, the grids were floated on a solution of protein A conjugated with 10 nm coloidal gold particles (Sigma, diluted 20-fold with TBS) for 1 h at room temperature. The samples were then rinsed and viewed directly or stained with 1% aqueous uranyl acetate and viewed in a JEOL transmission electron microscope.

3.3 RESULTS

To produce antibodies that specifically detect NS3, but not NS3A, in AHSV-3-infected cells, the N-terminal region unique to AHSV-3 NS3 was cloned into an antigen display vector, AHSV VP7mt200. The resulting VP7-NS3 chimera was expressed as a baculovirus recombinant and the hydrophobicity profile, solubility and crystal structure of the protein compared to the VP7mt200 vector and the wild-type AHSV-9 VP7 protein from which the vector was derived. Purified VP7-NS3 was used for the production of polyclonal antiserum and the resulting serum analysed in terms of its ability to bind AHSV-3 NS3.

3.3.1 Cloning of N-terminal region of NS3 into VP7mt200

The aim here was to make a recombinant construct, VP7-NS3, that would allow for the expression of the 12 N-terminal amino acids of NS3 on the surface of VP7 crystals (Fig 3.1). The insertion mutant VP7mt200 was chosen as a vector for immunological display as position 200 is believed to be exposed on the surface of the VP7 protein and the insertion of restriction enzyme sites at this site has been shown not to alter the ability of the protein to assemble into hexagonal crystals (Maree 2000). Cloning of a 36 bp region of the AHSV-3 NS3 gene into the *Xba*I/*Sal*I sites of VP7mt200 should therefore result in the expression of the first 12 amino acids of NS3 as an insert at position 200 on the surface of VP7.

Briefly, this entailed the directional cloning of nucleotides 20 – 55 of the AHSV-3 NS3 gene, encoding amino acids 1-12, between the *Xba*I and *Sal*I sites of the vector VP7mt200 cloned into the pFastBac plasmid (Fig 3.1). Plasmid DNA was extracted from possible recombinant *E. coli* colonies and insertion verified by *Pvu*I digestion (Fig. 3.2). The pFB-7mt200 vector has a single *Pvu*I site, digestion with this enzyme therefore results in the linearisation of the 5942 bp plasmid (Fig 3.2, lane b). The NS3-specific sequence contains a further *Pvu*I recognition site so that the insertion of this region into pFBmt200 and subsequent *Pvu*I digestion would result in two bands of approximately 2328bp and 3650bp (Fig 3.2, lanes c and d). Plasmids in which the NS3 sequence was not inserted would therefore be linearised by *Pvu*I digestion (Fig 3.2, lanes e and f). Note that incomplete digestion was achieved in lanes b and c. Automated sequence analysis of

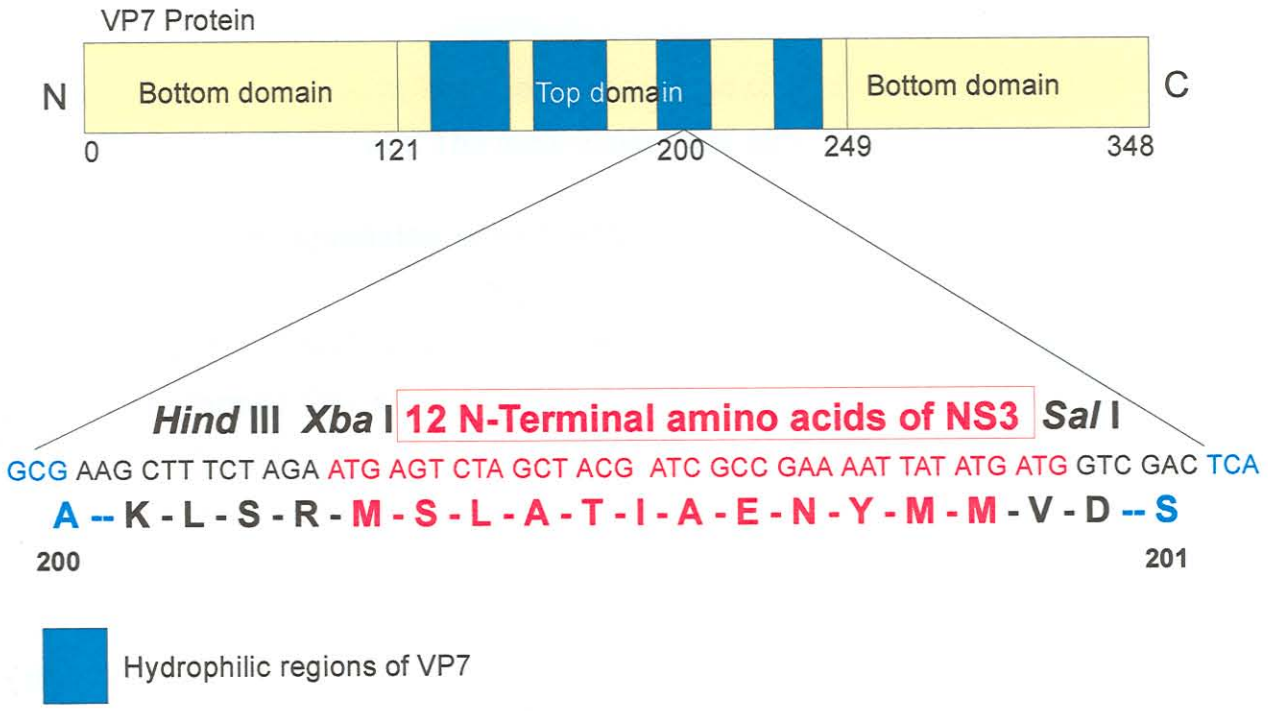


Figure 3.1 Schematic representation of the recombinant VP7-NS3 protein. A 36 bp region of the AHSV-3 NS3 gene (red) was cloned into the XbaI/SalI sites (black) of the VP7mt200 vector (yellow and blue). This would result in the expression of the first 12 amino acids of NS3 (red) as an insert at position 200 in a hydrophilic or surface region (blue) in the top domain of AHSV-9 VP7.

the recombinant plasmids verified the presence and correct sequence of the NS3 insert in VP7-NS3 (results not shown). The recombinant was termed pFBVP7-NS3.

3.3.2 Baculovirus expression of VP7-NS3

The pFBVP7-NS3 plasmid identified above was used to prepare a recombinant baculovirus, termed BACVP7-NS3, that would express the VP7-NS3 chimera in insect cells. Recombinant baculoviruses expressing the wild-type AHSV-9 VP7 protein (BAC-s9VP7) and the mutated version, VP7mt200 (BAC-VP7mt200), were also prepared for use as controls in all subsequent experiments. Plaque purified and amplified baculovirus stocks were used to infect *Sf9* cells and protein expression confirmed by analysis under a light microscope. Chuma and co-workers (1992) demonstrated that VP7 forms distinct disc-shaped crystals that are visible under a light microscope, when expressed as a baculovirus recombinant. Between one and three distinct morphological structures were visible in BAC-s9VP7, BAC-VP7mt200 and BACVP7-NS3-infected *Sf9* cells 2 days post infection. No such structures were visible in mock or wild-type infected cells. Crude cell lysates were prepared 72 h.p.i. and protein expression analysed by SDS-PAGE. The results are shown in Fig 3.3 (A). The predicted molecular weight of VP7-NS3 is approximately 42 kDa based on its amino acid sequence. A unique band corresponding to this expected size was detected in lane f, which represents BACVP7-NS3 infected *Sf9* cells (Fig 3.3A). The protein was larger than VP7mt200 (39 kDa, Fig 3.3, lane e) and VP7 (38 kDa, Fig 3.3A, lane d) as expected. The viral origin of the VP7 and modified VP7 proteins was confirmed by a Western blot with anti-AHSV-9 serum (not shown).

The presence of the NS3-specific insert in VP7-NS3 was confirmed by Western blots with anti-β-gal-NS3 and anti-NS3 sera. Neither the VP7 nor the VP7mt200 proteins reacted with these sera, while the VP7-NS3 chimeric protein reacted with the sera directed against either prokaryotic or eukaryotic expressed NS3. Fig 3.3(B) shows the results obtained with the anti-β-gal-NS3 serum. No immune reaction was observed in BAC-VP7 (Fig 3.3, lane d) or BAC-VP7mt200 (Fig 3.3, lane e) infected cells. A distinct protein band of 42 kDa, representing the VP7-NS3 chimera, was, however, observed in

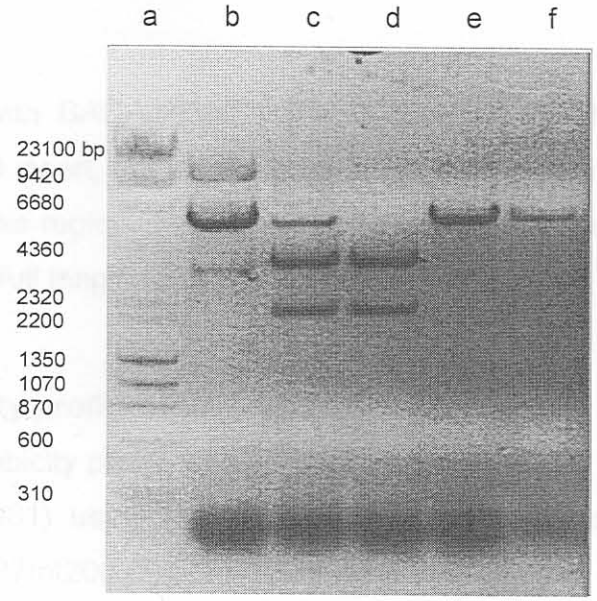


Figure 3.2 Agarose gel electrophoretic analysis of *Pvu*I digestion of possible pFBVP7-NS3 recombinants (lanes c – f). *Pvu*I linearised pFBmt200 was included as a control (lane b). Molecular weight markers (lane a) were *Hae*III-digested ΦX174 and *Hin*dIII-digested λ DNA.

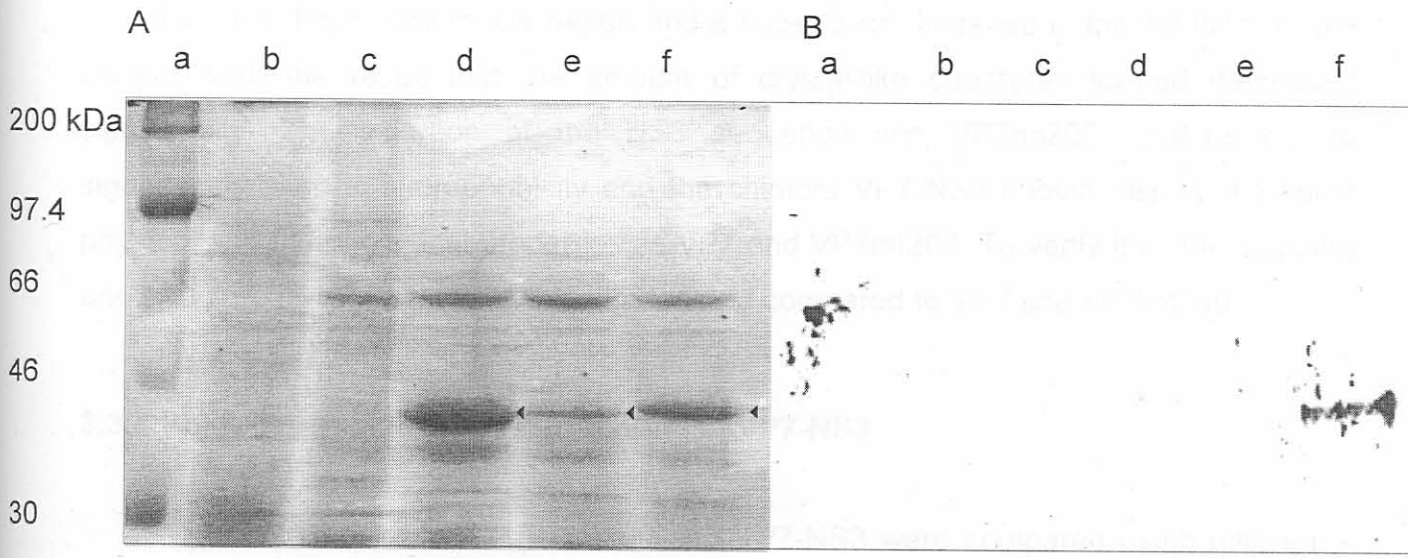


Figure 3.3 SDS-PAGE analysis (A) and Western blot (B) of recombinant baculovirus expressed VP7-NS3. *Sf*9 cells were mock-infected (b) or infected with wild type (c), VP7 (d), VP7mt200 (e) and VP7-NS3 (f) using recombinant baculoviruses. Cultures were harvested 72 h.p.i, separated by SDS-PAGE and stained with Coomassie brilliant blue (A) or blotted and reacted with anti-β-gal-NS3 serum (B). Rainbow protein markers were molecular weight markers (a). Arrows in A indicate the position of AHSV-9 VP7 (d), VP7mt200 (e), and VP7-NS3 (f).

Sf9 cells infected with BAC-VP7-NS3 (Fig 3.3B, lane f). This not only confirmed the presence of the NS3 insert, but also indicated that the polyclonal anti-NS3 sera contained antibodies against this region. This region of NS3 appears, therefore, to be immunogenic when present in the full length NS3 protein.

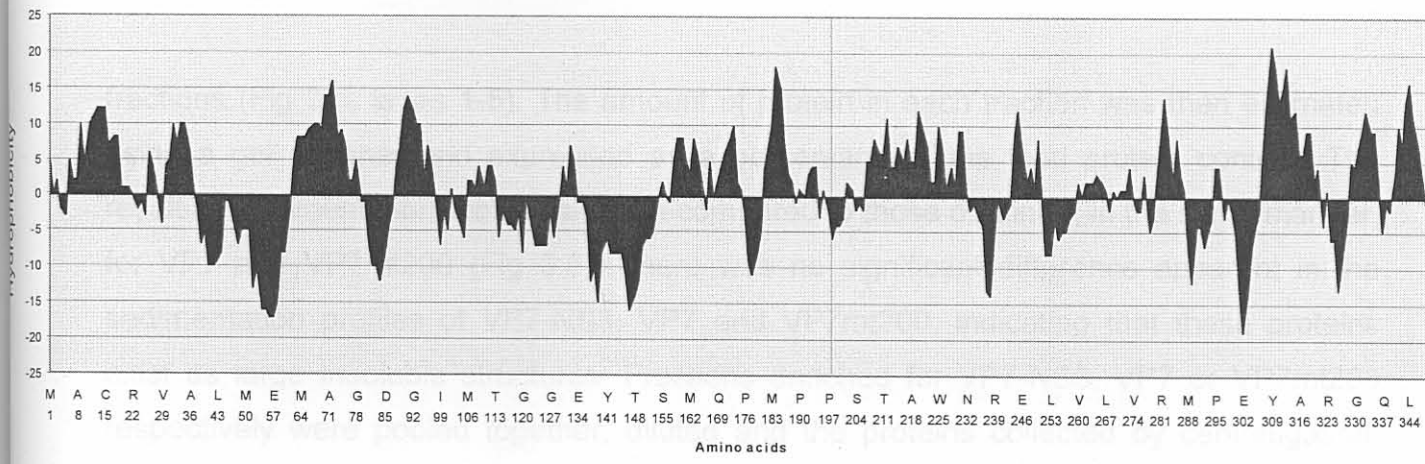
3.3.3 Hydrophobicity profile of VP7-NS3

The hydrophobicity profile of VP7-NS3 was determined according to the method of Hopp & Woods (1981) using the ANTHEPROT package, and compared to those of AHSV-9 VP7 and VP7mt200 (Fig 3.4). Excluding the region of insertion, the profiles of the VP7 proteins were identical. The profile of the inserted NS3 region in VP7-NS3 corresponded to the profile of this region in AHSV-3 NS3 (Van Staden, 1993), with a net increase in hydrophobicity at this site. Maree (2000) showed that the insertion of six amino acids (KLSRVD) between residue 177 and 178 of VP7 (VP7mt177) caused a large increase in hydrophilicity in this region and a subsequent increase in the solubility of the protein, with the result that the amount of crystal-like structures formed decreased significantly. The insertion of the NS3 sequence into VP7mt200 appears not to significantly alter its hydrophobicity and the chimera VP7-NS3 should display the same physical and morphological properties as VP7 and VP7mt200. To verify this, the solubility and structure of VP7-NS3 was investigated and compared to VP7 and VP7mt200.

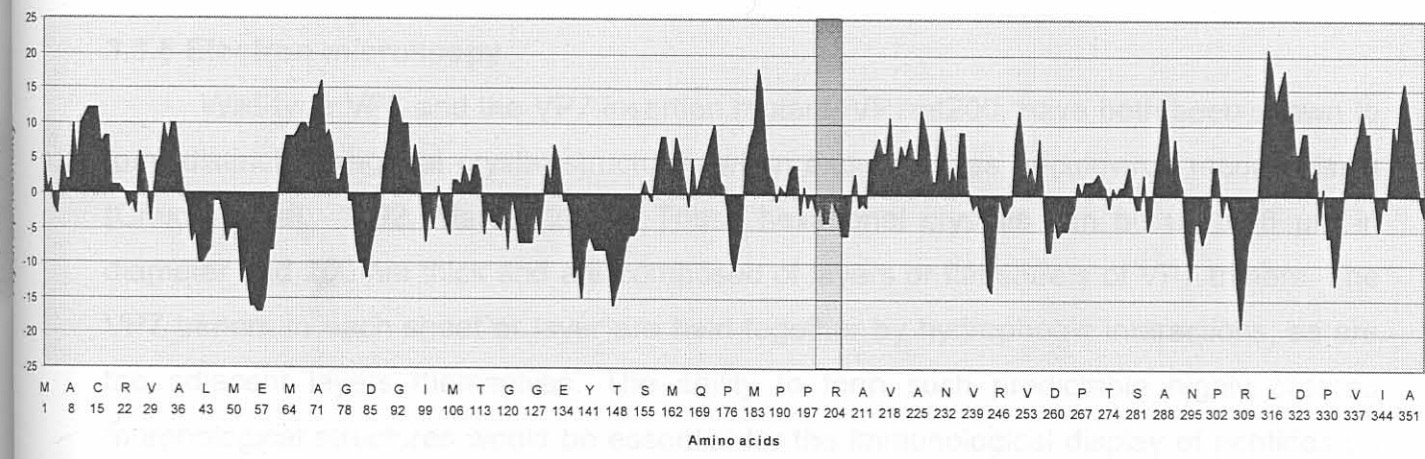
3.3.4 Solubility analysis and purification of VP7-NS3

The solubility of VP7, VP7mt200 and VP7-NS3 were compared using differential centrifugation and sedimentation analysis. Low speed centrifugation was used to separate cytoplasmic *Sf9* cell extracts containing these proteins into particulate and soluble fractions as outlined in 3.2.4. SDS-PAGE analysis revealed that, like VP7 and VP7mt200, VP7-NS3 was present predominantly in the particulate form (not shown). The particulate fractions were subsequently fractionated further by one-step sucrose gradient (40 – 75%) centrifugation and analysed by SDS-PAGE. Fig 3.5 illustrates the results obtained with the VP7-NS3 protein. Approximately 70% of the VP7-NS3 protein was present in the 5 bottom

(A) Hydrophobicity profile of VP7



(B) Hydrophobicity profile of VP7-200



(C) Hydrophobicity profile of VP7-NS3

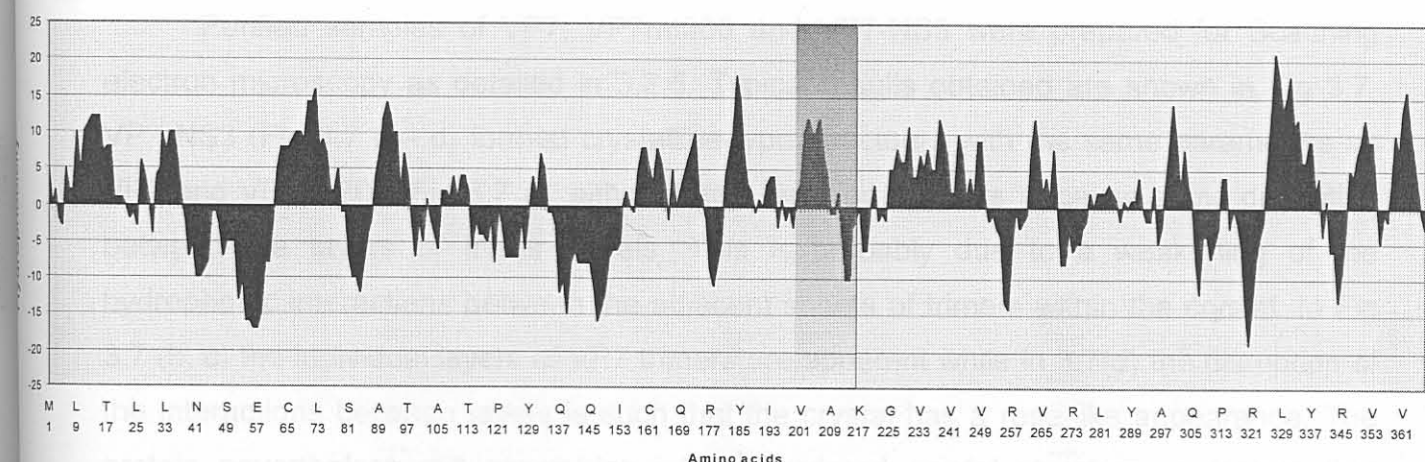


Figure 3.4 Hydrophobicity profiles of AHSV-9 VP7 (A), VP7mt200 (B) and VP7-NS3 (C), displaying regions with a net hydrophobicity (positive value) or hydrophilicity (negative value). Shaded regions represent the sites of insertion.

fractions (Fig 3.5, lanes 1-5). The amount of protein in each fraction was then estimated using a gel scanner and expressed as a percentage of the total protein content. The resulting sedimentation profile was then compared to those obtained, in the same manner, for VP7 and VP7mt200 (Fig 3.6). There was no significant difference apparent in the sedimentation profiles of VP7-NS3, VP7 and VP7mt200, indicating that these proteins exist as large insoluble structures. Fractions enriched for VP7-NS3, VP7 or VP7mt200 respectively were pooled together, diluted and the proteins collected by centrifugation. Proteins purified in this way were analysed by SDS-PAGE (not shown) and used for electron microscopical analysis and antiserum production.

3.3.5 Electron microscopy

Wild-type VP7 and the VP7 insertion mutant, VP7mt200, have both been shown to form distinct hexagonal crystal structures when expressed as baculovirus recombinants (Chuma *et al.,* 1992, Maree, 2000). These hexagonal crystals can be up to 6 µm in diameter and 200 nm thick and are composed of layers or flat sheets of VP7 trimers. The VP7 trimers in each sheet or layer are held together by hydrophobic interactions, as are the adjacent layers themselves. The ability to form such predictable highly ordered morphological structures would be essential for the immunological display of peptides on the surface of this protein. The ability of the VP7-NS3 chimera to form these typical structures was, therefore, investigated here.

Purified samples of VP7, VP7mt200 and VP7-NS3 were prepared for Scanning electron microscopy as detailed in 3.2.5. Typical results obtained are shown in Fig 3.7. VP7-NS3 (Fig 3.7 b – d) formed crystalline-type structures with the same parameters as VP7 and VP7mt200 (Fig 3.7 a), although the insertion appears to cause some disruption between the layers of trimer sheets. This is probably due to a weakening of the hydrophobic interactions between the adjacent sheets of trimers within the crystal. In Fig 3.7 (b, c) the individual layers of VP7 trimers are apparent while in 3.7(d) the disruption of the interactions between layers is such that the crystal has a rose-like appearance. The protein nevertheless still assembles into an ordered crystal structure, a feature that appears to be important in the immune response against AHSV VP7. This is based on the

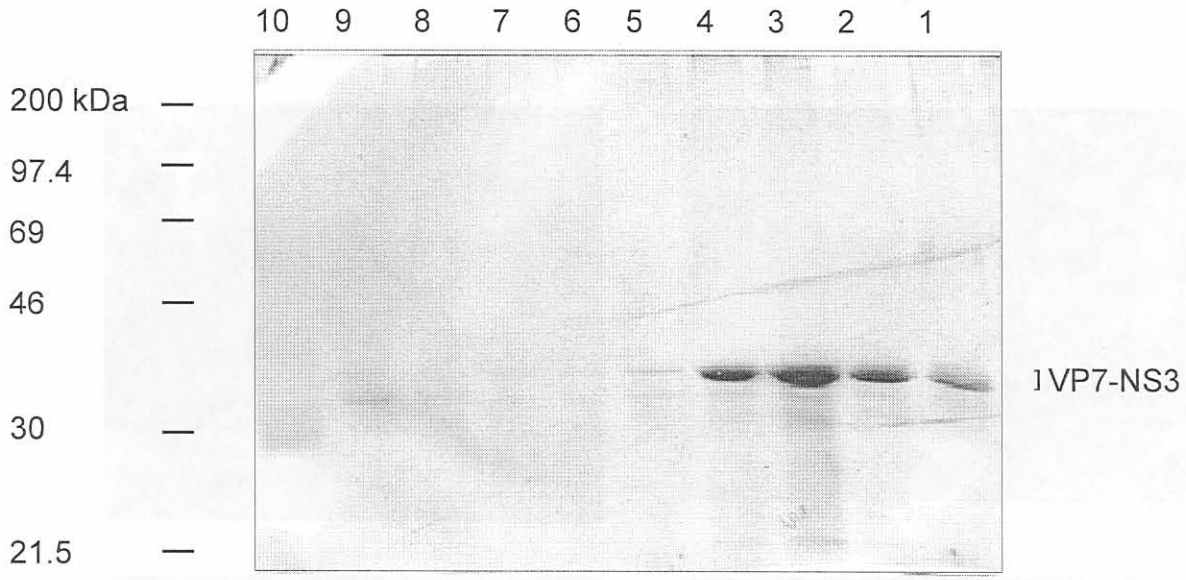


Figure 3.5 SDS-PAGE analysis of subcellular sucrose gradient fractionation of particulate fractions from *Sf*9 cells infected with a baculovirus recombinant expressing VP7-NS3. Fractions were collected from the bottom (1) to the top (10) of the gradient, analysed by SDS-PAGE and Coomassie blue staining. The molecular weight of the Rainbow size markers are indicated. The position of VP7-NS3 is indicated.

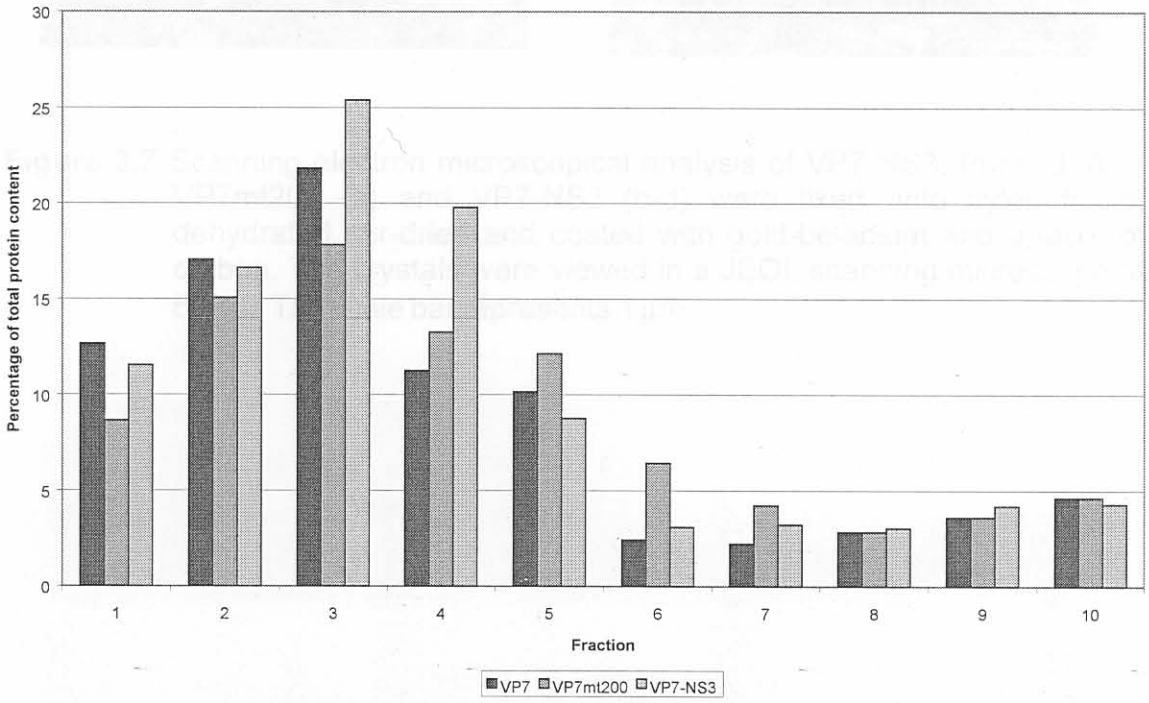


Figure 3.6 Comparison of sedimentation profiles of AHSV-9 VP7, VP7mt200 and VP7-NS3.

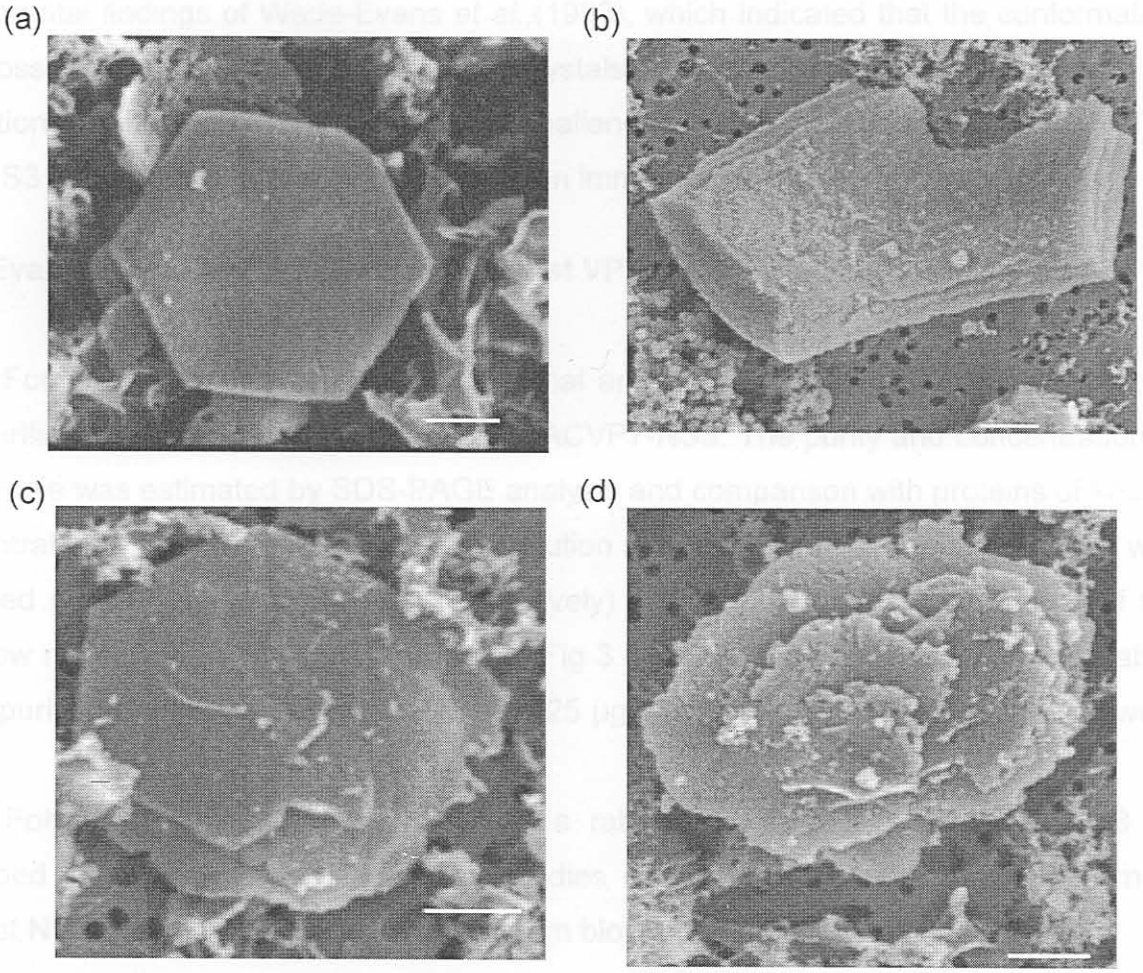


Figure 3.7 Scanning electron microscopical analysis of VP7-NS3. Purified VP7, VP7mt200 (a) and VP7-NS3 (b-d) were fixed onto nylon filters, dehydrated, air-dried and coated with gold-beladium and a layer of carbon. The crystals were viewed in a JEOL scanning microscope at 5.0 kV. The scale bar represents 1 μm.

experimental findings of Wade-Evans *et al.*,(1998), which indicated that the conformation and possibly the assembly of VP7 into crystals was important in the mechanism of protection against heterologous serotype challenge in mice immunised with VP7. The VP7-NS3 crystals should therefore still elicit an immune response.

3.3.6 Evaluation of antiserum raised against VP7-NS3

For the purpose of producing polyclonal antibodies against VP7-NS3, the protein was purified from insect cells infected with BACVP7-NS3. The purity and concentration of the sample was estimated by SDS-PAGE analysis and comparison with proteins of known concentration (Fig. 3.8). A 1x, 5x and 10x dilution series of the VP7-NS3 preparation was prepared (Fig 3.8, lanes b, d and f respectively) and compared to 1 µg of each of the Rainbow molecular weight marker proteins (Fig 3.8, lanes a, c and e). The concentration of the purified protein was estimated to be 0.25 µg/µl and no contaminating proteins were visible.

Polyclonal antiserum was raised in a rabbit against the purified VP7-NS3 as described in 3.2.6. The presence of antibodies directed against the N-terminal amino acids of NS3 was then evaluated by a Western blot and immunoprecipitation.

In a Western blot analysis with the anti-SfVP7NS3 antiserum (Fig 3.9) a single non-specific band was detected in mock (a), wild-type (b), BacVP7-NS3 (c), Bac-NS3 (d) and Bac-NS3A (e) infected cells. Several non-specific bands were present in all the lanes (excluding mock) that were not detected when the pre-inoculation serum was tested (not shown). These bands probably represent contaminating proteins co-purified with VP7-NS3. A single unique protein of the correct size (42 K) was detected in VP7-NS3 infected cells (Fig 3.9, lane c) and presumed to be VP7-NS3. The lane containing purified VP7-NS3 (Fig 3.9 lane f) contained a single band at the same position as this unique band, thereby confirming that it was VP7-NS3. No unique band corresponding to NS3 (24 K) was visible in the Bac-NS3 infected cells (Fig 3.9, lane d). The Western blot was repeated with similar results, and it was concluded that the antiserum did not contain antibodies directed against the linearised form of the N-terminal of NS3, only against VP7 epitopes.

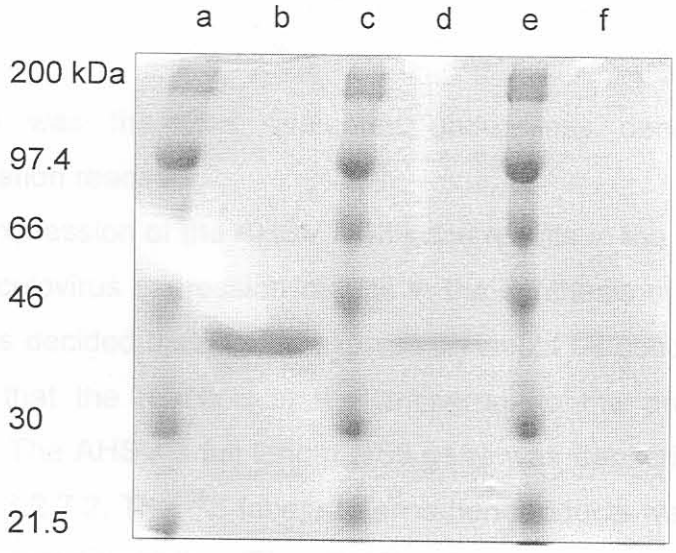


Figure 3.8 SDS-PAGE estimation of concentration and purity of the VP7-NS3 preparation for immunisation. A dilution series of purified VP7-NS3 (b,d,f) was compared to 1 µg of each of the Rainbow molecular weight marker proteins (a,c,e) by SDS-PAGE and Coomassie Blue staining.

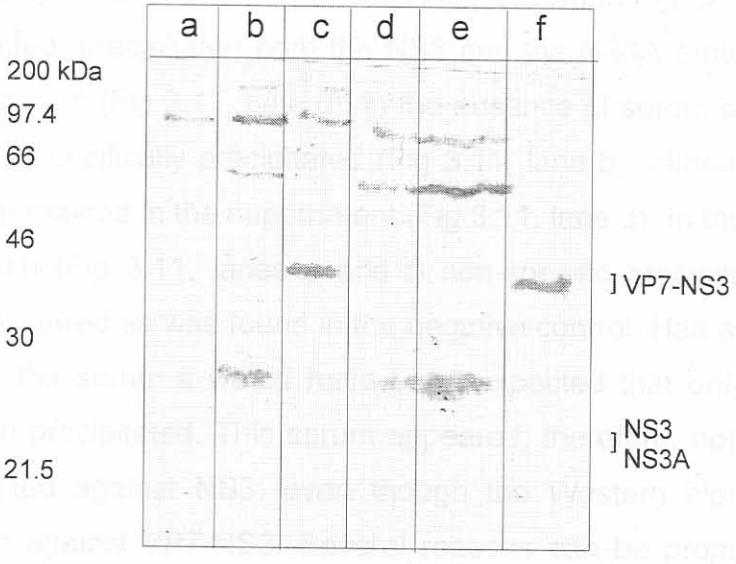


Figure 3.9 Western blot analysis of rabbit anti-SfVP7-NS3. *Sf*9 cells were mock infected (a) or infected with wild-type baculovirus (b), BACVP7-NS3 (c), BAC-NS3 (d), and BAC-NS3A (e). Purified VP7-NS3 was included as a control (f). Proteins were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes for reaction with the anti-Sf-VP7-NS3 serum. The position of the VP7-NS3 protein (c,f) and the expected position of NS3 (d) are indicated. The molecular weights of the rainbow markers are indicated on the left.

The antiserum was therefore evaluated under less denaturing conditions in an immunoprecipitation reaction.

In vitro expression of the AHSV NS3 gene results in the synthesis of both NS3 and NS3A while baculovirus expression results in the synthesis of NS3 only (Van Staden *et al.*, 1995). It was decided that *in vitro* expression would be used for the production of NS3 and NS3A so that the reaction of the antiserum to the proteins could be analysed simultaneously. The AHSV-3 full length NS3 gene was transcribed and translated *in vitro* as described in 3.2.7.2. The ^{35}S-labeled translation products were analysed by 15% SDS-PAGE and autoradiography (Fig. 3.10). As expected, two unique proteins of approximately 24 K and 23 K were synthesised, presumed to be NS3 and NS3A (Fig 3.10 lanes b,c,d). A translation reaction without the NS3 mRNA was performed as a negative control (Fig 3.10 lane a) and neither of these two proteins was detected in this lane.

The NS3 and NS3A proteins were precipitated in the presence of rabbit anti-SfVP7-NS3 and anti-β-gal-NS3 (positive control). As a negative control a precipitation reaction was set up without any antiserum. The results are shown in Fig. 3.11. The anti-β-gal-NS3 serum, as expected, precipitated both the NS3 and the NS3A proteins (Fig 3.11, lane d) out of the supernatant (Fig 3.11, lane c). In the absence of serum a small amount of both proteins was non-specifically precipitated (Fig 3.11, lane b), although the majority of the NS3 and NS3A remained in the supernatant (Fig 3.11, lane a). In the presence of the anti-SfVP7-NS3 serum (Fig 3.11, lanes e and f) non-specific precipitation of the NS3 and NS3A proteins occurred as was found in the negative control. Had antibodies against NS3 been present in the serum it would have been expected that only NS3 and not NS3A would have been precipitated. This serum appeared, therefore, not to contain antibodies specifically directed against NS3, even though the Western blot clearly indicated an immune reaction against VP7-NS3. Several reasons can be proposed to explain these findings and will be discussed in the following section.

One possible reason could be that the NS3 sequence was not, in fact, exposed on the surface of the VP7 crystal. In an attempt to investigate this the purified VP7-NS3 crystals were labeled with the anti-β-gal-NS3 serum, that gave a positive reaction in the Western blot (3.3.2), and then reacted with a secondary antibody labeled with gold particles (3.2.8). Purified VP7mt200 crystals were labeled in the same manner as a

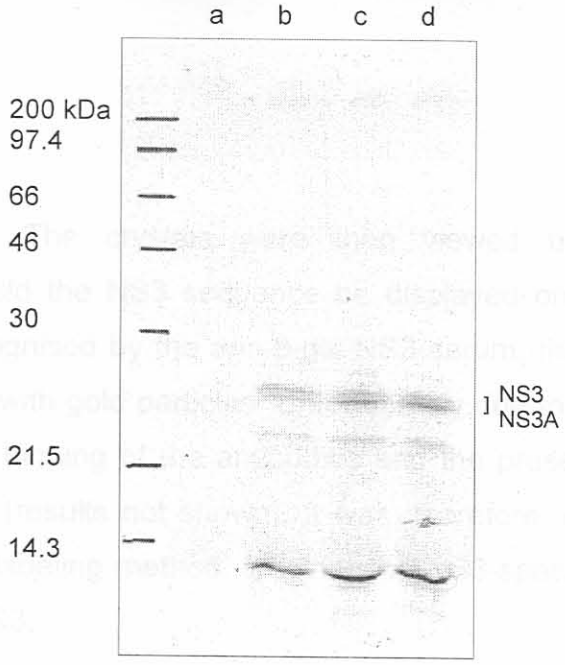


Figure 3.10 Analysis of *in vitro* translation of mRNA from *in vitro* transcribed pBS-NS3. Varying amounts of mRNA were used as templates and the ^{35}S labeled translation products (b,c,d) analysed by 15% SDS-PAGE and autoradiography. The positions of NS3 and NS3A are indicated. As a negative control RNA was excluded from the reaction (a). Rainbow molecular weight markers are indicated.

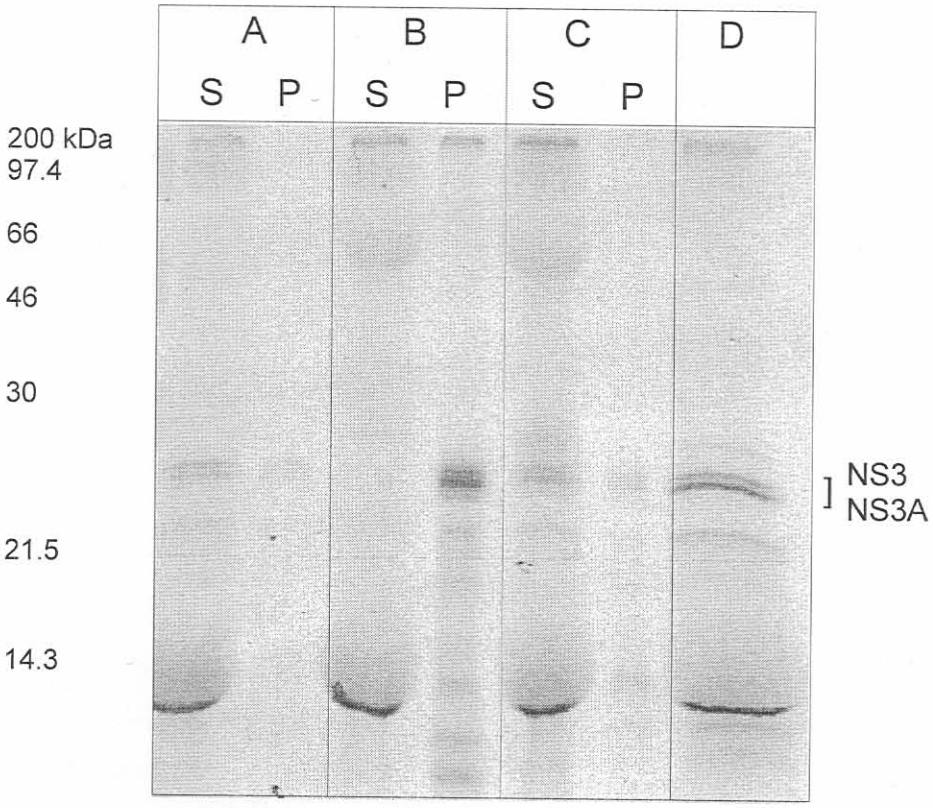


Figure 3.11 Immunoprecipitation analysis of *in vitro* translated AHSV-3 NS3 and NS3A. ^{35}S-labeled translation products were precipitated in the absence of serum (A) and in the presence of anti-β-gal-NS3 (B) or anti-SfVP7-NS3 (C). Unprecipitated proteins in the supernatant (S) and precipitated proteins in the pellet (P) after centrifugation were analysed by 15% SDS-PAGE and autoradiography. *In vitro* translated NS3 and NS3A were included as a control (D) and the positions of NS3 and NS3A are indicated. Rainbow molecular weight markers are indicated.

negative control. The crystals were then viewed under a transmission electron microscope. Should the NS3 sequence be displayed on the surface of VP7-NS3, in a conformation recognised by the anti-β-gal-NS3 serum, then the crystal would have been uniformly labeled with gold particles. Unfortunately due to the extreme electron density of the VP7 crystals, binding of the antibodies and the presence of the gold particles could not be visualised (results not shown). It was, therefore, not possible to ascertain, using this immunogold labeling method, whether the NS3-specific insert was displayed on the surface of VP7-NS3.

3.4 DISCUSSION

Proteins encoded from the same ORF from in-frame start codons, like NS3 and NS3A of AHSV, may be functionally distinct as a result of, for example, a domain in the N-terminal region of the full length protein. Alternatively the absence of the N-terminal extension in the smaller protein(/s) may result in the exposure of a functional domain. These functional domains could, in both cases, control the localisation, or even the activity or specificity of the protein. The S and L surface antigens of Hepatitis B virus, for example, have been shown to be functionally distinct (Oess & Hildt, 2000, see section 1.6.2b), while the P1-P5 proteins of Rabies virus have been shown to be differentially localised (Chenik *et al.,* 1995, see section 1.6.2c).

Comparison of the localisation of the NS3 and NS3A proteins of AHSV would require not only a system capable of monitoring the synthesis and transport of these proteins through the cell, but also a means of differentiating between these almost identical proteins. Antibodies that are directed against the N-terminal of NS3 would provide a means of detecting NS3 in AHSV infected cells. Using these antibodies, in techniques such as immunogold labelling and electron microscopy of infected cells, the ultrastructural localisation of NS3 and NS3/A could be compared. This could potentially also facilitate the identification of cell or virus components that are associated with NS3 and/or NS3A. Alternatively AHSV-infected cells could be fractionated and the presence of NS3 and/or NS3A investigated in each fraction. This would then allow possible insight into a distinct role for these two related proteins in the viral life cycle. Antibodies directed against the N-terminal of NS3 may furthermore be used to experimentally analyse the orientation of the NS3 protein in the cell membrane. The orientation of the influenza virus M2 protein was determined in this way (Lamb *et al.,* 1985). The topology of the NS3 protein has not yet been experimentally elucidated.

Polyclonal antibodies directed against the 12 N-terminal amino acids of NS3 could be prepared against a synthetic peptide representing this region. Peptides are usually synthesized using the solid-phase techniques pioneered by Merrifield in 1963 (Harlow & Lane, 1988). Because of their size, peptides may not be immunogenic on their own. To elicit an antibody response directly, they must contain all of the features of an immunogen,

notably they must have an epitope for B-cell binding and a site for class II-T-cell receptor binding. Some peptides, even surprisingly small ones, contain both these sites usually as a single sequence that can serve both these functions (Harlow & Lane 1988). Unfortunately, there are no methods, short of immunisation, to test this. Most peptides are therefore coupled to carrier proteins prior to immunization. Typical carrier proteins include keyhole limpet hemacyanin (KLH) and bovine serum albumin (BSA). The carrier protein provides good sites for class II-T-cell receptor binding and the peptide itself can be seen as an epitope. Using this approach high-titered antibodies are commonly prepared that characteristically bind well to denatured proteins, but may or may not recognise the native protein (Harlow & Lane, 1988). Alternatively the synthetic peptide could be coupled to a synthetic class II-T-cell receptor (Harlow & Lane, 1988) or to a promiscuous T-cell epitope (Frangione-Beebe *et al.,* 2000). The central problems to this type of strategy are the fact that the native protein is often not recognised by the antisera produced and that the synthesis of peptides is costly.

An alternative would be to clone the DNA sequence encoding the peptide into the surface region of a structural protein that has a predictable high-order particulate structure and that is immunogenic. This type of strategy has become quite common in, for example, particulate vaccine strategies. In fact the VP7 protein of AHSV is currently being engineered for this purpose as it has the characteristics of an antigen display vector (Maree 2000). The protein is immunogenic and spontaneously assembles into a high-order structure. VP7 crystals purified from BHK cells infected with AHSV-9 were shown to be highly immunogenic, eliciting a strong immune response and effective as a subunit vaccine in a mouse model (Wade-Evans *et al.,* 1997, Wade-Evans *et al.,* 1998). Passive transfer of antibodies from immunised mice failed to protect syngeneic recipients from AHSV challenge, indicating that an antibody response is unlikely to represent the primary mechanism involved. It is possible that immunisation with VP7 induces an initial protective T-cell response. Furthermore, the conformation and possibly the assembly of VP7 into crystals appear to be important in the mechanism of protection against heterologous serotype challenge (Wade-Evans *et al.,* 1998). VP7 of BTV, furthermore, contains immunodominant, serotype cross-reactive T-cell epitopes (Wade-Evans *et al.,* 1998). An extension mutant of BTV VP7 containing 48 amino acids of Hepatitis B virus preS2 region

at the N-terminus was found to be highly immunogenic, although the results indicated that the suitability of the N-terminus of BTV VP7 for the insertion of foreign epitopes was relatively restricted due to instability (Belyaev & Roy, 1992; Le Blois & Roy, 1993).

Regions on the AHSV VP7 top or exposed domain have been identified and targeted for the insertion of unique restriction enzyme sites for the cloning and display of foreign epitopes. The insertion of these cloning sites between residues 200 and 201 were shown not to alter the ability of the protein to assemble into hexagonal crystals (Maree, 2000). This insertion mutant, VP7mt200, should be an ideal vector for antigen display. It was therefore decided to use this vector, VP7mt200, for the immunological display of the NS3 N-terminal region as this would simultaneously allow for the production of antibodies against NS3 and for the testing of this protein as an antigen display vector, as this has not previously been done.

Insertion of the NS3 sequence into VP7mt200 was achieved by the synthesis of oligonucleotides representing the N-terminus of NS3 and cloning of this into the *Xba*I and *Sal*I sites at position 200 of pFB-7mt200. The hydrophobicity profile of the amino acid sequence of VP7 remained unaltered by the insertion of the NS3 sequence. Expression of the VP7-NS3 chimera as a baculovirus recombinant resulted in a unique protein of the correct size (± 42 K). This protein reacted positively in an immunoblot with anti-NS3 and anti-β-gal-NS3 sera, indicating that the chimera did contain the NS3 sequence. This furthermore indicated that the N-terminal region of NS3 was immunogenic and should give some immune reaction in VP7-NS3. The solubility and sedimentation profile of VP7-NS3 were compared to the native VP7 protein and the VP7mt200 protein, and found not to differ significantly, remaining highly hydrophobic and insoluble. The VP7-NS3 protein was purified and viewed under a scanning electron microscope.

AHSV VP7 and VP7mt200 aggregate into flat, hexagonal crystals (Roy *et al.,* 1991; Chuma *et al.,* 1992; Burroughs *et al.,* 1994; Maree, 2000). These crystals are composed of layers or flat sheets of VP7 trimers. In each layer of the crystal the trimers are arranged as hexagonal rings, similar to the rings of VP7 trimers found on the surface of AHSV core particles (Burroughs *et al.,* 1994; Basak *et al.,* 1996; Grimes *et al.,* 1998; Stuart *et al.,* 1998). These layers are believed to be held together by hydrophobic interactions, similar to the interactions between VP7 and the underlying VP3 molecules in the core particles.

The VP7-NS3 chimera produced here appears to still form the sheets of trimers although the hydrophobic interactions between these sheets appears to be disrupted, so that the crystals no longer appear flat and the individual layers of trimers are apparent. In extreme cases the crystals have a rose-like appearance. The VP7-NS3 protein, nevertheless, still forms a highly ordered structure and should still be suitable for antigen display.

Monospecific polyclonal antibodies were raised against purified VP7-NS3 crystals. The antiserum produced recognised VP7-NS3 although no specific reaction with denatured or non-denatured AHSV-3 NS3 was detected. Antibodies directed against the NS3 N-terminal may be present in the serum but at a negligibly low titre. This may be due to immunodominance, where the antibody response against one compound, in this case the VP7 epitopes, dominates the response when the immunogenicity of that compound is higher (Harlow & Lane, 1988). The NS3 sequence, which has a net hydrophobic charge, may have folded inwards in such a way that it was not exposed on the surface of the crystal and therefore not accessible to the environment. Alternatively the peptide could not have been exposed on the surface of the crystal simply due to its small size. This could be tested by immunogold labelling of VP7-NS3 crystals using the anti-β-gal-NS3 serum that reacted with this protein in a Western blot. This was attempted, but the extreme electron density of the crystals prevented any clear indication of antibody binding. This could also be tested by immunofluorescent labelling of the crystals in infected cells and viewing with a fluorescent microscope.

A model of the three-dimensional structure of an AHSV VP7 trimer is shown in Fig 3.12 (Maree, 2000), sites that have been targeted for insertion are indicated (VP7mt144, blue, VP7mt177 green and VP7mt200 red). From this Figure it can be seen that position 200 is less exposed on the surface and although insertions at this site do not alter the hydrophobicity, solubility or crystal structure of the protein, the alternative sites (144, 177) may represent better targets for the display of such a small peptide. Conversely tandem repeats of the NS3 sequence could be cloned into the VP7mt200 vector, with a greater probability of being exposed on the surface of the resulting crystals.

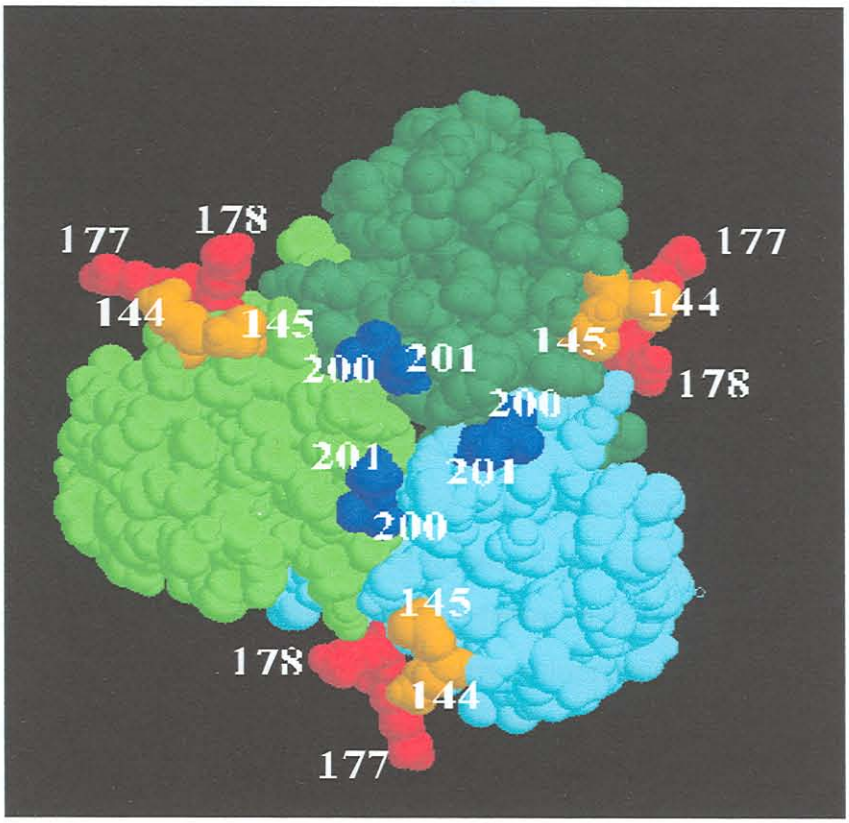


Figure 3.12 Model of the VP7 trimer 3-D structure indicating mutational insertions that have been made at hydrophilic regions exposed on the surface of the protein. The backbone of the VP7 protein is indicated in green and the insertion sites 144-145, 177-178 and 200-201 are indicated in orange, red and navy blue respectively (Maree, 2000).

The insertion of six amino acids at position 177-178 of VP7 (recombinant VP7mt177) caused a significant increase in the solubility of the VP7 protein with the result that only a fraction of the protein formed morphological structures. The structures formed by VP7mt177 were not the distinct hexagonal crystals observed with wild type VP7, but small ball-like structures which only later, when examined with a transmission electron microscope, were shown to possess an ordered crystalline lattice (Maree, 2000). This insertion site is more exposed on the surface of the VP7 crystal (Fig 3.12) and may be a more suitable vector for the display of a small peptide considering it has now been shown to posses a highly ordered structure.

A 105 amino acid region of AHSV VP2 has been inserted into VP7mt200 and VP7mt177. These chimeric proteins were expressed as baculovirus recombinants and found to have an increased solubility. These chimeras no longer formed the typical hexagonal crystals but did however appear to form multiprotein structures (Maree, 2000). The immunogenicity of these chimeras has not been investigated.

The use of AHSV VP7 as an antigen display vector definitely still merits further investigation. The use of viral particulate structures for antigen or epitope display has been successful in a number of cases. Heal and coworkers (1999), for example, modified the coat protein of the RNA bacteriophage MS2 for the display of antigens as a novel vaccine strategy. The coat protein of MS2 self-assembles into capsids that elicit both humoral and cellular immune responses. The N-terminus of this protein contains a beta-hairpin loop that forms the most radially exposed feature of the mature capsid. This site was targeted and modified to enable insertion of DNA at the central part of the beta-hairpin loop for the display of foreign peptides on the surface of RNA-free bacteriophage capsids. A putative protective epitope, T1, from the immunodominant liver stage antigen-1 (LSA-1) of the malaria parasite *Plasmodium falciparum* was subsequently cloned into this site. The recombinant construct was expressed in *E. coli* and the immunogenicity of the resulting chimeric capsids was found to correspond well to naturally acquired resistance to liver stage malaria (Heal *et al.,* 1999).

A similar type of approach was also taken with the development of an antigen presentation system based on the plum pox potyvirus (PPV). The amino-terminal part of PPV capsid protein was chosen as the site for the expression of foreign antigenic

peptides. Different forms of an antigenic peptide (single or tandem repeats) from the VP2 capsid protein of canine parvovirus (CPV) were expressed at the chosen site. Mice and rabbits immunised with purified chimeric virions developed CPV-specific neutralising antibodies (Fernandez-Fernandez *et al.,* 1998). Other examples of particulate vector systems for the presentation of immunogenic epitopes include recombinant BTV core-like particles (Mikhailov *et al.,* 1996), BTV NS1 tubules (Mikhailov *et al.,* 1996) and hepatitis B virus core protein (Chambers *et al.,* 1996).

To summarize, the AHSV core protein VP7 was used as an antigen presentation vector for the display of the N-terminal region of NS3. The chimeric protein assembled as a highly-ordered crystal structure and was used for the production of polyclonal serum. Although no antibodies against AHSV-3 NS3 were obtained in this study, the VP7 vector is still in its developmental stages and the use of alternative sites on VP7 are proposed for the display of such a small peptide.