

**Tubules composed of non-structural protein  
NS1 of african horsesickness virus as a  
system for the immune display of foreign  
peptides**

by

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## SUMMARY

### **TUBULES COMPOSED OF NON-STRUCTURAL PROTEIN NS1 OF AFRICAN HORSESICKNESS VIRUS AS A SYSTEM FOR THE IMMUNE DISPLAY OF FOREIGN PEPTIDES**

by

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For the degree MSc

Non-structural protein, NS1 of *African horse sickness virus* is a hydrophobic protein of 63 kDa that spontaneously assembles into highly distinct tubular structures when expressed in mammalian or insect cells. The spontaneous assembly of these proteins into a predictable multimeric structure, high levels of expression and ease of purification make this protein an ideal candidate for the immune display of foreign peptides. The potential of such a display system has been investigated for BTV NS1 that is able to successfully elicit both a humoral and a cellular immune response against inserted peptides. The aims of this study were to investigate both the stability of the AHSV NS1 particulate structure after insertion of peptides as well as the antigenicity and immunogenicity of the peptides presented in this system.

Two overlapping regions consisting of 40 and 150 amino acids, and which correspond to a neutralising region identified within the AHSV major neutralising protein VP2, were inserted into an internal site in NS1. This site offered the best surface display of inserted peptides on the tubular structures. An enhanced green fluorescent protein, 240 amino acids long, was also inserted into the NS1 protein.

Sucrose gradient analysis of the recombinant proteins indicated that the majority of the baculovirus expressed chimeric proteins formed particulate structures with a sedimentation value similar to that of the native NS1 protein. This was confirmed by transmission electron microscopic analysis, which clearly showed that all the chimeric

proteins assembled into tubular structures similar to those observed for AHSV NS1 proteins. Furthermore, fluorescence analysis of sucrose gradients of NS1/eGFP also showed high levels of fluorescence that corresponded directly to particle formation. Not only do the inserts remain functional but are also presented successfully on the surface of the intact NS1 tubule structure.

The potential of the NS1 vector to efficiently present peptides to the immune system was subsequently investigated. The serums generated against these chimeric proteins in guinea pigs were tested against chimeric constructs, the baculovirus expressed inserts (for eGFP) and the inserts presented on other presentation vectors. Western blot analysis showed that most of the serums generated against the chimeric proteins contained antibodies not only against the chimeric proteins but antibodies that reacted specifically with the inserted peptides on their own or on another presentation system. Preliminary immune studies seem to indicate that the humoral immune response elicited by the chimeric NS1 proteins is predominantly against the inserts. The inserts are successfully presented to the immune system on the surface of the NS1 vector and are able to elicit the production of antibodies with the potential to provide a protective immune response.

## LIST OF ABBREVIATIONS

AcNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
AHS	African horsesickness
AHSV	African horsesickness virus
AHSV9	African horsesickness virus serotype 9
AHSV6	African horsesickness virus serotype 6
AIDS	Acquired immunodeficiency syndrome
APSV	Afrika perde siekte virus
AP	Alkaline phosphatase
APC	Antigen presenting cells
bp	Base pairs
BSA	Bovine serum albumin
BT	Bluetongue
BTV	Bluetongue virus
°C	Degrees Celsius
cDNA	Complementary DNA
CLP	Core-like particles
Cm <sup>2</sup>	Square centimeter
CFA	Complete Freund's adjuvant
CTL	Cytotoxic T lymphocyte
Da	Dalton
DNA	Deoxyribonucleic acid
ddH <sub>2</sub> O	Deionized distilled water
dNTP	Deoxyribonucleoside-triphosphate
EDTA	Ethylenediaminetetra-acetic acid
EHDV	Epizootic hemorrhagic disease virus
<i>et al.</i>	et alia ( and others)
FCA	Freunds complete adjuvant
FCS	Fetal calf serum
FIA	Freunds incomplete adjuvant
FIV	Feline immunodeficiency virus
Fig.	Figure

FMD	Foot and Mouth Disease
FMDV	Foot and mouth disease virus
g	Gram
HBV	Hepatitis B virus
HbsAg	Hepatitis B virus surface antigen
HSV	Herpes simplex virus
h	Hour
HIV	Human immunodeficiency virus
IFA	Incomplete Freund's adjuvant
IgA	Immunoglobulin class A
IgG	Immunoglobulin class G
IPTG	Isopropyl-D-thiogalactopyranoside
KDa	Kilodalton
kb	Kilobasepairs
l	Liter
LB	Luri Bertani
M	Molar
Mab	Monoclonal antibody
MCS	Multiple cloning site
mg	Milligram
MHC 1	Major histocompatibility complex 1
MHC 11	Major histocompatibility complex II
min	Minutes
ml	Milliliter
mM	Millimolar
MOI	Multiplicity of infection
M <sub>r</sub>	Molecular weight
ng	Nanogram
NS	Nonstructural
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PGLA	Poly-D-L-lactide-glycolide
pmol	Picomol
<i>polh</i>	Polyhedrin
PSB	Protein solvent buffer
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SIV	Simian immunodeficiency virus
TC	Tissue culture
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris-hydroxymethyl-aminomethane
µg	Microgram
µl	Microliter
UV	Ultraviolet
V	Volts
VLP	Virus like particle
VP	Viral protein
wcf	Whole cell fraction
w	Weight
X-gal	5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

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## CHAPTER 1

### LITERATURE REVIEW

#### 1.1. GENERAL INTRODUCTION

A new age in disease control dawned with the introduction of Jenner's cowpox virus as a vaccine for the prevention of smallpox. Ever since that discovery vaccination has constituted the most cost-effective tool for the prophylaxis of infectious diseases and the diligent use of measles, diphtheria, tetanus and pertussis vaccines have been associated with the dramatic reduction in the incidence of these diseases worldwide. In the case of smallpox, the dream of eradication has been fulfilled. Equally important has been the development in veterinary medicine of vaccines to control leptospirosis, rinderpest and foot-and-mouth disease in cattle, clostridial diseases in sheep, cattle and pigs, diarrhea caused by *Escherichia coli* in piglets and Newcastle and Marek's disease in poultry (Brown, 1992).

Vaccines will play an even more critical role in the future as the rapid increase in urbanisation and farming are resulting in the crowding of people and animals, thereby ensuring the rapid spread of infection not only between individuals of the same species, but between species. This point is underlined by the emergence of Nipah virus where the virus was transmitted from fruit bats to humans and pigs (Parasher *et al.*, 2000). The emergence of HIV and the resurgence of traditionally third world diseases like tuberculosis have also led to a renewed interest in vaccine development. An even greater concern is a pandemic caused by some newly evolved strain of influenza that epidemiologists have warned could sicken one in every three people on the planet and lead to ten to hundreds of millions of deaths (Guan *et al.*, 2002; Ito *et al.*, 1998; Li *et al.*, 2004; Peiris *et al.*, 2004; Webster *et al.*, 1992). These warnings are not unfounded as many communities in Asia live in close proximity to aquatic birds such as ducks and geese that are natural hosts for influenza (Chen *et al.*, 2004; Chen *et al.*, 2005; Hulse-Post *et al.*, 2005; Webster, 2004). Several cases have already reported where strains of avian flu have killed people in Asia and are moving towards Europe (Chan, 2002; Chotpitayasunondh *et al.*, 2005; Tran *et al.*, 2004; Yu *et al.*, 2006). Although this strain, influenza A subtype H5N1 does not yet effectively transmit from person to person, the virus is evolving (Ungchusak *et al.*, 2005). Even though H5N1 is susceptible to the commercially available antiviral drugs, oseltamivir and zanamivir (marketed under the brand names Tamiflu and Relenza) based on the neuraminidase inhibitors, either drug must be taken within 48 hours of symptoms being observed (De Clercq, 2005; Govorkova *et al.*, 2001; Gubareva *et al.*, 1998; Jefferson *et al.*, 2006; Leneva *et al.*, 2001).

Unfortunately the vaccine strategies used for the control of smallpox and polio will not work against influenza unless major advances in vaccine technology are achieved. Based on all these factors, more emphasis will have to be placed on the prevention of infectious diseases through new vaccine strategies and less emphasis on post-infection treatment.

The evolution of vaccine strategies has seen a move from whole organisms, such as attenuated or inactivated pathogens, to recombinant proteins and further towards the ultimate in minimalist virology, the epitope. A greater understanding of immunity to infectious pathogens and advances in the identification of protective antigens on a range of pathogens, together with developments in molecular and cell biology, have enabled a more rational approach to the development of safer subunit vaccines, consisting of specific antigenic components of the pathogen (McNeela, Mills, 2001). Besides the classical whole-cell vaccines, consisting of killed or attenuated pathogens, new vaccines based on the subunit principle, have been developed, e.g. the Hepatitis B surface protein vaccine and the *Haemophilis influenza* type b vaccine (Plotkin, 1993).

In the following chapter the basic principles of immunology, relevant to the development of vaccines and specifically AHSV vaccines, will be discussed as well as the associated advantages and problems of the three existing vaccine approaches namely conventional whole-cell vaccines, subunit vaccines and nucleic acid vaccines.

As this study focuses on the development of the AHSV NS1 protein as a novel platform for the presentation of antigens, this discussion briefly reviews AHSV and related viruses in the of context epidemiology, molecular biology and structure and disease control. The discussion will specifically focus on AHSV NS1 as presentation system.

## **1.2. AFRICAN HORSE SICKNESS VIRUS**

*African horsesickness virus* (AHSV) is classified as a member of the genus *Orbivirus* in the family *Reoviridae* (Bremer, 1976; Holmes, 1991; Murphy *et al.*, 1995; Oellermann *et al.*, 1970; Verwoerd *et al.*, 1979). This virus is similar in morphology, physiochemical and immunological properties to other orbiviruses such as *Epizootic haemorrhagic disease virus* (EHDV), *Equine encephalosis virus* (EEV) and

*Bluetongue virus* (BTV) with BTV being the prototype virus of this genus (Spence *et al.*, 1984; Verwoerd *et al.*, 1979).

African horse sickness (AHS) was first described by Theiler in 1921 as a “disease of equines caused by an ultraviolet virus, probably transmitted by a blood-sucking insect” (Theiler, 1921). According to Theiler (1921) the first reference to AHS was made by father Monclaro in 1569 and then again in 1719 in the official documents of the Cape of Good Hope in which “perreziekte” was identified as killing 1700 horses. In actual fact the earliest known reference to a disease resembling AHS is in an Arabian document that documents the disease in the Yemen in 1327 (Coetzer, Erasmus, 1994). Theiler correctly predicted the infectious agent and “insect theory” of transmission for AHS and he also described the four clinical forms of AHS based on symptomatology.

The disease is manifested by symptoms such as pyrexia, inappetence, and clinical signs such as lesions compatible with impaired respiratory and circulatory functions that are characterized by oedema of subcutaneous and intramuscular tissues and of the tissues of the lungs, transduction into body cavities and hemorrhages. Four clinical forms of AHS are distinguished based on symptoms of the infected animal, namely horse sickness fever form, pulmonary or peracute forms (Dunkop), cardiac subacute form (Dikkop) and mixed or acute form. These four forms are associated with 0%, 95%, 50% and 50 to 95% mortality in horses, respectively (Brown, Dardiri, 1990; Burrage, Laegreid, 1994; Carrasco *et al.*, 1999; Coetzer, Erasmus, 1994; House, 1993; Theiler, 1921).

AHS occurs predominantly in sub-Saharan Africa and is endemic to a band stretching from Senegal in the west to Ethiopia and Somalia in the east and extends as far as southern Africa. The virus is limited to areas where vectors for disease transmission is present (Brown, Dardiri, 1990; House, 1993; Howell, 1963; Mellor, Boorman, 1995). The disease is transmitted between susceptible animals by biting midges, specifically *Culicoides imicola* (Du Toit, 1944) but Meiswinkel *et al.* reported in 2000 that in certain high-lying regions of South Africa *Culicoides bolitinos* may be the major vector of the disease (Meiswinkel *et al.*, 2000). The disease has also been reported in donkeys, zebras, dogs (Barnard, 1997; Barnard *et al.*, 1994; Braverman, Chizov-Ginzburg, 1996; Brown, Dardiri, 1990; Mellor, 1994) and antibodies against AHSV has been reported in cheetahs, lions, African wild dogs, jackals, hyenas, large-spotted genets and free-living elephants (*Loxodonta africana*) (Alexander *et al.*,

1995; Barnard *et al.*, 1995). Most of these animals, with the exception of domestic dogs and possibly wild dogs, are regarded as poorly susceptible and unlikely to be a source of AHSV. The role of these animals in the epizootology of AHSV infection is uncertain but questions have been raised about whether these animals could potentially spread the virus (Alexander *et al.*, 1995; Hamblin *et al.*, 1998). It has been suggested that zebra are the most likely reservoir of AHSV, but there is no definitive answer (Barnard, 1998).

### 1.2.1. The Genome

The genomes of all orbiviruses contain 10 segments of double-stranded RNA, with the exception of Colorado tick fever that contains 12 segments (Huisman *et al.*, 1979; Knudson *et al.*, 1984; Kusari, Roy, 1986; Oellermann, 1970). The segments are numbered in the order of migration and is grouped as large (L1-3), medium (m4-6) and small (S7-10) (Roy *et al.*, 1994). Each of the genome segments have a distinct size on PAGE gel and a unique profile can be distinguished for each of the nine AHSV serotypes (Bremer *et al.*, 1990).

Most of the genome segments of AHSV code for one protein with the exception of segment 1 that codes for VP1 and a protein with a slightly lower molecular weight, segment 6 that codes for VP5 and VP6 and segment 10 that codes for NS3 and NS3a (Grubman, Lewis, 1992). Each of these genome segments are transcribed individually and the segments are functionally distinct. Some inconsistencies exist in the literature for the AHSV coding assignments of segments 5, 6, 9 and 10. It has been reported that segment 5 encodes non-structural protein NS1 (Grubman, Lewis, 1992; Maree, Huisman, 1997), segment 6 codes for VP5 and VP6 (Grubman, Lewis, 1992; Roy *et al.*, 1994), segment 9 codes for NS3 and segment 10 codes for NS3 and NS3A (Grubman, Lewis, 1992). According to O'Hara *et al.* (1993) *in vitro* translation data that these assignments are incorrect and that in actual fact segments 5, 6 and 9 encode VP5, NS1 and VP6, respectively (O'Hara *et al.*, 1993). For the purpose of this study it will be assumed that AHSV6 segment 5 codes for NS1 based on the findings of Maree and Huisman (1997). Segment 10 encodes two non-structural proteins, NS3 and NS3a, from two different overlapping reading frames (van Staden, Huisman, 1991; van Staden *et al.*, 1995). Seven of the genome segments code for a single structural protein (Mizukoshi *et al.*, 1993), while the remaining three encode non-structural proteins (Wu *et al.*, 1992). The protein and genome coding assignments for AHSV are shown in Table 1.

**Table 1:** AHSV proteins and genome coding assignments (Grubman, Lewis, 1992; Maree, Huismans, 1997; Vreede, Huismans, 1994).

Genome segment	Serotype	Segment length (bp)	Protein	Predicted Molecular Weight (Da)	Protein length (aa)
1	9	3965	VP1	150 292	1305
2	3	3221	VP2	123 063	1057
3	4	2795	VP3	103 269	905
4	4	1978	VP4	75 826	642
5	9	1566	VP5	505	56 771
5	6	1748	NS1	548	63 478
6	9	1748	NS1	548	63 377
7	9	1167	VP7	349	37 916
8	9	1166	NS2	365	41 193
9	3	1169	VP6	369	38 464
10	9	756	NS3/NS3A	217/ 206	23 659/ 22481

### 1.2.2. The Virion And Associated Proteins

AHSV proteins can be classified into two groups, namely structural and non-structural proteins, based on whether the proteins form part of the virus structure or are just virus-specified protein products.

#### 1.2.2.1. The structural proteins

The intact AHSV is formed by seven structural proteins that can be grouped as outer capsid proteins (VP2 and VP5), major core proteins (VP3 and VP7) and the minor virion proteins (VP1, VP4 and VP6) (Bremer, 1976; Bremer *et al.*, 1990; Els, Verwoerd, 1969; Le Blois *et al.*, 1991; Roy *et al.*, 1994; Verwoerd *et al.*, 1972). The major core proteins and minor proteins form the inner capsid or core particle that encloses the dsRNA genome. VP7 trimers form the knob-like protrusions on the surface of the core and overlays a scaffold of VP3 (Grimes *et al.*, 1995; Hewat *et al.*, 1992b; Hewat *et al.*, 1992c; Prasad *et al.*, 1992). The core particle is surrounded by an outer capsid layer that consists of VP2 and VP5 that appears to form continuous layers around the core. The sail-shaped VP2 forms protruding spikes that are situated above the VP7 trimers (Hewat *et al.*, 1992c).



It is thought that the minor core proteins (VP1, VP4 and VP6) are involved in enzymatic functions such as virus replication and transcription. BTV VP1, the largest of the proteins, is the putative virion RNA polymerase (Roy *et al.*, 1988; Urakawa *et al.*, 1989). In BTV it has been shown that VP4 is involved with mRNA capping of viral mRNA and methylation during transcription and it is possible that VP4 acts as a guanylttransferase (Mertens *et al.*, 1992). VP6 has been shown to have a strong binding affinity for single- and double-stranded RNA and has been identified as a putative helicase involved in the unwinding of the viral double-stranded RNA genome (Mertens *et al.*, 1992; Roy *et al.*, 1990a; Roy *et al.*, 1994). Studies on AHSV VP2 support the identity of this protein as a putative helicase (Turnbull *et al.*, 1996).

## **VP2**

The structural protein, VP2 is encoded by segment 2, the largest segment of the capsid genome (Grubman, Lewis, 1992; Roy *et al.*, 1994). The AHSV9 VP2 gene is 3205 bp long and encodes a protein 1053 amino acids (Venter *et al.*, 2000). This protein, together with VP5, forms the outer capsid layer of the AHSV particle and the sail-shaped VP2 forms protruding spikes that are situated above the VP7 trimers (Hewat *et al.*, 1992c). This outer capsid protein is most variable amongst serotypes and serogroups and is the principal serotype-specific antigen (Fukusho *et al.*, 1987; Huismans, Erasmus, 1981; Iwata *et al.*, 1992b; Vreede, Huismans, 1994). It is not unexpected then that the neutralizing epitopes of AHSV is located on VP2 (Burrage *et al.*, 1993; Martinez-Torrecuadrada, Casal, 1995; Vreede, Huismans, 1994). BTV VP2 is thought to be the viral haemagglutinin and is directly involved in attachment of virus to cells (Cowley, Gorman, 1987; Eaton, Crameri, 1989).

### **1.2.2.2. The non-structural proteins**

Four non-structural virus proteins, NS1, NS2, NS3 and NS3A, are synthesized in orbivirus infected cells, but do not form part of the virus structure (Gorman *et al.*, 1981; Huismans, 1979). Relatively little is known about the function and role of these proteins in the orbivirus infection cycle, but it is generally thought to function during replication.

NS2 is the only viral phosphorylated protein and is closely associated with the formation of virus inclusion bodies observed in virus infected cells and the protein synthesis of NS2 corresponds to that of virus inclusions bodies (Devaney *et al.*, 1988; Huismans, Basson, 1983; Huismans *et al.*, 1987b; Theron *et al.*, 1994). The

fact that orbivirus NS2 can bind single-stranded RNA (Huismans, Basson, 1983; Huismans *et al.*, 1987b; Theron *et al.*, 1994; Thomas *et al.*, 1990; Uitenweerde *et al.*, 1995) and that inclusion bodies are sites at which viral assembly occurs has led to the idea that NS2 may be involved in the recruiting of double stranded RNA during the virus assembly process (Thomas *et al.*, 1990). This idea is supported by the finding of Theron *et al.* (1994) that phosphorylation of NS2 seems to down regulate its single stranded RNA binding ability.

The two smallest non-structural proteins, NS3 and NS3A, are translated from two in-frame translation initiation sites in genome segment 10 (French *et al.*, 1989; Mertens *et al.*, 1984; Van Dijk, Huismans, 1988; van Staden, Huismans, 1991; Wu *et al.*, 1992). These proteins are thought to be responsible for virus release from infected cells (Hyatt *et al.*, 1991; Hyatt *et al.*, 1993; van Staden, Huismans, 1991; van Staden *et al.*, 1995; Wu *et al.*, 1992).

### **NS1**

The non-structural protein NS1 forms characteristic tubules in the cytoplasm of orbivirus infected cells and are characteristically associated with sites of virus maturation (Huismans, Els, 1979; Murphy *et al.*, 1971; Thomas, Miller, 1971; Tsai, Karstad, 1973a; Tsai, Karstad, 1970; Verwoerd *et al.*, 1979). The tubules are present in large numbers in predominantly peri- or juxtannuclear locations and a proportion of the tubules are associated with the cellular cytoskeleton (Eaton, Hyatt, 1989; Eaton *et al.*, 1987; Hyatt, Eaton, 1988). Based on the observation that virus particles leaving the virus inclusion bodies are associated with NS1 it has been suggested that NS1 may play a role in virus morphogenesis (Eaton *et al.*, 1987). If soluble NS1 plays a role in viral morphogenesis the condensation of NS1 into tubules may occur after the critical concentration of soluble NS1 has been reached, but it is more likely that NS1 monomers very rapidly assemble into tubules (Nel, Huismans, 1991). It has been suggested that the tubules themselves do not have a functional role and if the soluble NS1 monomers play a role in the infection cycle a vast amount of NS1 has to be synthesized to compensate for the high rate of tubule self assembly (Nel, Huismans, 1991). In a study by Venter *et al.* (1991) a large excess of NS1 mRNA was detected in EHDV-, AHSV- and BTV-infected cells very early in the infection cycle and Huismans (1979) reported BTV tubules as early as 2-4 hours post infection and in large numbers at 6 hours post infection (Eaton *et al.*, 1990; Huismans, 1979; Lewanczuk, Yamamoto, 1982; Venter *et al.*, 1991). In terms of function this is an

important indicator that NS1 plays a role soon after infection and is required in large amounts.

Although no function has been ascribed to the NS1 tubule, the theory up to very recently was that the tubules are involved in virus particle translocation to the plasma membrane. This theory was largely based on the finding that NS1-specific monoclonal antibodies react with virus particles that are in close proximity to inclusion bodies or that are exiting from infected cells (Eaton *et al.*, 1990). In a recent study by Owens *et al.* (2004) it was shown that NS1 tubules may in actual fact play a direct role in the cellular pathogenesis and virus release of BTV (Owens *et al.*, 2004). In this study the effect of NS1 tubule disruption on BTV replication was monitored by introducing a NS1 monoclonal antibody specific to the carboxy terminus of the protein (Monastyrskaya *et al.*, 1995). The carboxy terminus of NS1 is required for tubule formation and binding of the carboxy-terminus-specific NS1 monoclonal antibody inhibited tubule formation. Inhibition of tubule formation in BTV infected mammalian cells resulted in a reduction in virus-induced cytopathic effect, an increased release of infectious virus into the culture medium and budding of virions from the plasma membrane. In the presence of NS1 tubules, BTV infection of cultured mammalian cells results in severe cytopathic effect within 24 to 48 hours post infection characterised cell rounding, apoptosis and lytic release of infectious virus into the culture medium. Based on these results it was proposed that the NS1 protein plays a major role in the pathogenesis in the vertebrate host and that it plays a role in the augmentation of virus-cell association and not the transport of the virus to the cell surface as previously thought. It must also be said that that BTV-induced cellular pathogenesis might be a function of the relative ratio of NS1 tubule levels to NS3 protein levels within the cytoplasm of infected cells, but this hypothesis has yet to be tested (Owens *et al.*, 2004).

NS1 is encoded by genome segment 5 (Maree, Huismans, 1997; Nel *et al.*, 1991; Urakawa, Roy, 1988) and in the case of AHSV6 it encodes a 548 aa polypeptide, 63 478 Da in size (Huismans, Els, 1979; Maree, Huismans, 1997). The protein has a net charge of +5 at neutral pH and is a highly hydrophobic protein (Maree, Huismans, 1997). These hydrophobic domains are conserved in the NS1 proteins of AHSV, BTV, EHDV and BRDV (Maree, Huismans, 1997; Moss, Nuttall, 1995) and it is thought that these hydrophobic stretches are important for tubule formation, hence the conservation amongst NS1 proteins of orbiviruses.

Electron microscopic analysis of the AHSV6 tubule indicated that its fine structure differed from that of BTV, EHDV and EHDV (Moss, Nuttall, 1995; Nel, Huismans, 1991; Urakawa, Roy, 1988) in that it did not have the ladder like-surface structure with linear periodicity, but instead an internal 'cross-weave' appearance (Maree, Huismans, 1997) Baculovirus expressed AHSV6 tubules have a morphology similar to that of native tubules isolated from AHSV3 infected cells (Huismans, Els, 1979) with an average diameter of  $23 \pm 2$  nm, and tubules of varying lengths of up to  $4\mu\text{m}$  (Maree, Huismans, 1997). The AHSV tubules have the smallest diameter when compared to that of BTV NS1 tubules (68 nm) and EHDV tubules (Huismans, Els, 1979).

Although no three dimensional studies have been performed on AHSV NS1 tubules such studies exist for BTV (Hewat *et al.*, 1992a) and EHDV (Nel, Huismans, 1991). Hewat *et al.* (1992) showed that BTV tubules are composed of a coil ribbon of NS dimers with 22 dimers per helix turn. EHDV in comparison consists of approximately 16 subunit per turn (Nel, Huismans, 1991).

The formation of virus associated tubular structures have been reported in cells infected with reovirus (Stoltz, Makkay, 2000), Infectious Bursal Disease, a member of the *Birnaviridae*, (Granzow *et al.*, 1997), certain members of the Herpesviridae family (Diosi *et al.*, 1980; Ecob-Johnston, Whetsell, 1979), pig rotavirus (Pearson, McNulty, 1979) and other arthropod-borne orbiviruses such as Colorado tick fever (Murphy *et al.*, 1968; Oshiro, Emmons, 1968), Tribec (Gresikova *et al.*, 1969), Wad Medani, Chenudi, Irituia, Palyam, Lebombo (Borden *et al.*, 1971). It is interesting to note that majority of these tubular structures are usually similar in diameter to that of the virus particle, but at this stage the function of all of the virus associated tubules are unknown.

### **1.3. VACCINES**

#### **1.3.1. Principles Of Vaccinology**

All existing vaccines are based on the principle that recovery from an infection results in resistance to subsequent infection (Babiuk *et al.*, 1996). In order to be able to rationally design a vaccine it is necessary to understand the immune mechanisms that induce protection. Recent developments in our understanding of the pathways of immunity required to produce protection against different infections, allow immunological principles to be incorporated into the design of new and better vaccines (Griffin, 2002).

The immune system can be divided into two broad functional pathways: humoral immunity (HI) and cellular immunity (CI). Humoral immunity is mediated by antibody/immunoglobulin (Ig) molecules, which are produced by B cells under regulatory influence of T cells (Griffin, 2002; Shearer, Clerici, 1994). Antibodies bind to antigenic determinants, usually on the surface of viruses, bacteria or parasites, and inactivate these infectious agents (Shearer, Clerici, 1997). Hence, for most viral and bacterial infections, primary protection is mediated by a humoral immune response (production of antibodies) (Gurunathan *et al.*, 2000b). In the case of viruses, antibodies act by prevention of infection of cells, prevention of release of virus from cells (Yokomori *et al.*, 1992) opsonisation or complement-mediated lysis of the virus infected cells and by antibody-dependant cell-mediated cytotoxicity (Rouse *et al.*, 1976a; Rouse *et al.*, 1976b). Antibodies are important mainly in protection against reinfection, but they also assist in clearing an established infection (Bangham, Phillips, 1997).

Cell mediated immunity or cellular immunity forms the second arm of the immune system and results from the activation of effector mononuclear phagocytes or T cells, under the control of regulatory T cells. In contrast to HI, CI is mediated by effector cells that destroy infected cells of the host by direct cell-to-cell contact, or by the release of molecules that possess killing activity. In this way CI focuses on the destruction of the source of infection, and is more effective against intracellular infections. CI is less clearly understood than HI and appears to be more complex (Griffin, 2002; Shearer, Clerici, 1997). The cellular immune response comprises primarily CD4+ and CD8+ cells. These cells recognize foreign antigens that have been processed and presented by antigen presenting cells (APC) in the context of MHC class II or class I molecules, respectively (Gurunathan *et al.*, 2000a; Gurunathan *et al.*, 2000b; Gurunathan *et al.*, 2000c; Zinkernagel, Doherty, 1997).

The type of infection will determine which of these immune pathways will be activated and must be taken into consideration when designing a vaccine. The protective immune response to a pathogenic microorganism requires the induction of antigen-specific cells of the immune system and the subsequent generation of effector mechanisms. For most viral and bacterial infections, primary protection is mediated by a humoral response. For intracellular infection and other parasites, protection is mediated by cellular immunity; moreover, for some diseases both humoral and cellular responses are likely to be required.

RNA viruses, such as AHSV, present additional challenges to vaccine design in that they have a mutation rate of about  $1 \times 10^4$  to  $1 \times 10^6$  times greater than that of DNA viruses, because of RNA replicase lacking proof-reading capacity of DNA replicases. Viruses also utilize programmed rearrangements in parts of their genomes to evade recognition by the host's immune system. In addition to this, viruses with a RNA genome are capable of particularly rapid replication (Holland *et al.*, 1992) hence, producing vast numbers of viral particles. The combination of rapid replication and mutation gives RNA viruses the ability to evolve extremely quickly *in vivo*, producing mutants with antagonistic or partial antagonistic properties and so evade the immune system and antiviral drugs (Bangham, Phillips, 1997). The combination of these factors complicates the design of new vaccines, especially the development of subunit vaccines that usually focus on immunodominant areas of the virus genome. The rapid rate of replication of these viruses necessitates that any vaccine against these viruses elicit the correct initial immune response as the incorrect response will not fully protect the vaccinee against viral infection and will only afford partial protection.

Irrespective of which organism is involved, the purpose of immunization remains to increase the number of antibody-producing cells that can produce antibody and cells that can respond with a cell-mediated immune response. These responses are aimed to neutralize and limit the amount of damage and immunopathology produced by the interaction of the host and pathogen. As a result of infection or exposure to specific antigens of a pathogen, the number of specific cells capable of recognizing the same or related antigens is dramatically expanded. These 'memory' cells can then respond quickly to pathogen upon re-exposure of the host to the same or related pathogens. Therefore both humoral and cellular response occurs not only more rapidly, but also at a much higher level than following a primary infection (Bradley *et al.*, 1993; Kinman *et al.*, 1989). Thus, vaccination should maintain sufficient levels of antibodies and cytotoxic T cells in circulation to either prevent infection or rapidly limit the degree of replication.

In the case of AHSV, horses that recover from the first primary infection show complete immunity to homologous virus infection. At present the precise contribution of the humoral and cell-mediated immune response to protection and immunity is not clear, but it has been shown that there is a very strong humoral basis (Burrage, Laegreid, 1994). Studies by Alexander (1935), Burrage (1993), Blackburn (1988) and Stone-Marschat *et al.* (1996) suggests that neutralising antibodies are primarily

responsible for protection against AHSV and that the presence of neutralising antibodies correlate with protection against homologous viral serotypes (Alexander, 1935; Blackburn, Swanepoel, 1988; Burrage *et al.*, 1993; Stone-Marschat *et al.*, 1996). From this it is obvious that an effective AHSV vaccine must incorporate the epitopes that elicit neutralising antibodies in horses and any potential vaccine must be able to stimulate an effective immune response to those epitopes. VP2 and VP5 are the only AHSV and BTV proteins known to contain neutralising epitopes (Martinez-Torrecedrada, Casal, 1995; Martinez-Torrecedrada *et al.*, 1996; Martinez-Torrecedrada *et al.*, 1994; Martinez-Torrecedrada *et al.*, 1999; Ranz *et al.*, 1992; Roy *et al.*, 1996; Van Wyngaardt *et al.*, 1992; Vreede, Huismans, 1994). Although VP5 contains neutralising epitopes (Martinez-Torrecedrada *et al.*, 1999), VP2 is the major neutralising antigen (DeMaula *et al.*, 1993; Roy *et al.*, 1996) as VP5 is probably less exposed on virus surface than VP2 due to at least three strong hydrophobic domains on VP5 (Roy *et al.*, 1994). The effectiveness of the VP2 protein as a vaccine will depend on its ability to stimulate the production of large amounts of antibody against itself and this will depend on the inherent immunogenicity of the protein as well as other factors such as the delivery system and antigen presentation.

Not much information is available concerning the role of cell-mediated immunity during AHSV infection, but it is thought that cell-mediated immunity has an effect on limiting the virus infection as well as recovery from it (Burrage, Laegreid, 1994). Although most research indicates that immunity against AHSV is humoral based a study by Wade-Evans *et al.* (1997) showed that the AHSV outer core protein VP7 can elicit protection in mice against AHSV and that this response is unlikely to be due to antibody-mediated response (Wade-Evans *et al.*, 1997).

### **1.3.2. Classification Of Vaccines**

Vaccines can be divided into two broad groups: whole-cell vaccines and subunit vaccines. Whole-cell vaccines which form the majority of vaccines used today are usually produced by conventional methods using principles developed by Jenner and Pasteur just over 200 and 100 years ago, respectively. Although these vaccines have been and still are extremely successful in the control and prevention of diseases they are not without risks and there are serious diseases for which there are no available vaccines for. Subunit vaccines have the potential to overcome many of these problems, but it would be overly optimistic to think that this new approach could replace all existing successful whole-cell vaccines or control infections that can not be controlled by conventional vaccine strategies.

Before embarking on the development of a new vaccine all the positive and negative aspects of each vaccine approach should be weighed up against each other. In the following section it will be attempted to give an objective and unbiased overview of most of the existing vaccine development strategies.

### **1.3.2.1. Whole-cell vaccines**

Whole-cell vaccines consist of complete organisms and can be divided into live attenuated vaccines and killed/inactivated vaccines. Both of these types of vaccines have proven to be effective in at least partially reducing clinical manifestations following exposure to virulent field strains of pathogens. Further more they have helped curtail the spread of the pathogens to other individuals by reducing the quantity of pathogen shed into the environment.

#### **a. Live attenuated vaccines**

Live vaccines induce immunity by mimicking natural infections using attenuated forms of the pathogen. Its disease-causing capacity is eliminated by biological or technical manipulations while remaining sufficiently infectious to function as a vaccine.

Using conventional technology, attenuation is achieved by passaging the agent *in vitro* either in the presence of mutagenizing agents or under various *in vitro* culturing conditions. The vaccines used to control AHS and BT are polyvalent live attenuated vaccines. The vaccine used at present for the prevention of AHS is attenuated *in vitro* by serial passage of AHSV vaccine strains in Vero (African green monkey kidney) cell cultures (Coetzer, Erasmus, 1994; House *et al.*, 1992; House, 1998). Important examples of *in vitro* attenuated vaccines used for routine pediatric vaccination are polio (Sabin, 1985) and measles (Enders *et al.*, 1960). The licensed vaccine for preventing typhoid fever is an good example of live attenuated vaccine production by chemical mutagenesis where this method was applied to the Ty21a strain of *Salmonella typhi* (Levine *et al.*, 1987).

Wild type organism can also be genetically attenuated through the deletion of genes that potentiate virulence or insertion of attenuating genes (Babiuk *et al.*, 2002; Ellis, 2001). Deletion of genes that affect virulence produces a stably attenuated phenotype that cannot revert to virulence as in the case of Herpes simplex virus type mutant with an essential glycoprotein deleted (Farrel *et al.*, 1994) and impressive



efficacy has been observed for strains of simian immunodeficiency virus (SIV) with deletions in the *nef* gene (Ruprecht, 1999).

Typically, live attenuated vaccines induce broad based and long-lived immune responses (Ruprecht, 1999). Live vaccines usually elicit both humoral (antibodies) and cellular immunity (e.g. cytotoxic T-lymphocytes) (Ellis, 2001) and live attenuated vaccine virus cause an acute infection and induces both specific antiviral antibodies and T-cell immunity which limit virus propagation and ultimately clear the vaccine virus infection. Only a single or a few doses may give protection (Hansson *et al.*, 2000).

Two main drawbacks of attenuated vaccines are the risks of incomplete levels of attenuation and reversion into the original pathogenic forms, especially in immunocompromised individuals and infants (Appel, 1978; Minor *et al.*, 1986). Moreover, it is possible that some live vaccine strains can be transmitted from vaccine to an uninfected individual (Hansson *et al.*, 2000). There are also additional disadvantages, such as the presence of contaminating extraneous viruses grown in tissue culture (Babiuk *et al.*, 2002) and the fact that attenuated vaccines are alive requires the maintenance of a cold chain and this may be a real impediment in remote areas (Babiuk *et al.*, 2002). Another important factor concerns the immunization of infants where the presence of passive antibody from the mother will limit the replication and development of immunity to the vaccine. This leaves the young vulnerable to disease for extended periods of time (Kinman *et al.*, 1989).

Although effective, live vaccines have several serious disadvantages that need to be considered before embarking on this approach.

#### **b. Killed vaccines**

An approach used to overcome the problem of safety and reversion encountered with attenuated vaccines is the use of killed vaccines. Inactivated influenza A and B virus vaccines have been licensed for parenteral administration to humans for over 40 years (Wareing, Tannock, 2001). Killed vaccines are produced by inactivating the infectious agent through physical or chemical processes so that it cannot replicate in the host without altering the immunogenicity of protective proteins (Babiuk *et al.*, 2002; Hansson *et al.*, 2000). Inactivated vaccines are usually made by treating the agent with a chemical, such as formalin, to denature the toxin or to kill the agent. For current conventional inactivated vaccines against FMD the virus is activated by

testament with binary ethyleneimine and not formalin as this inactivation agent was associated with viral escapes (Barteling, Vreeswijk, 1991; Beck, Strohmaier, 1987).

This approach, though safer than attenuation, is associated with numerous disadvantages. Firstly, killed vaccines cannot replicate and are non-infectious, but are not very immunogenic; therefore the need for booster injections and strong adjuvants to improve efficacy. Another very important disadvantage is that killed vaccines usually function by stimulating the humoral immune response, as well as priming for immunological memory, but elicits limited, if any, cell-mediated immunity (Audibert, Lise, 1993; Bangham, Phillips, 1997). In terms of safety, even though the vaccine itself contains killed organisms, the production of such vaccines still requires the large-scale culturing *in vitro* of the disease-causing micro-organism, which can be associated with both safety risks and problems to achieve cost-effective production (Hansson *et al.*, 2000). For killed vaccines, great care must be taken to ensure that the agent is inactivated completely or it may cause disease in recipients (Dertzbaugh, 1998). In some instances, even after formalin inactivation there is a serious risk of reversion. In some instances field outbreaks have occurred as a consequence of incomplete inactivation. Another drawback of attenuated or killed whole-cell vaccines is that the present requirements from regulatory agencies, e.g. the Food and Drug Administration (FDA) and the World Health Organization (WHO), for exact specifications of the vaccine composition and mechanisms to obtain immunity, are difficult to meet (Liljeqvist, Stahl, 1999).

For these reasons and others, new approaches are being considered for vaccine development, which are not based on the whole organism. These include the use of: (1) recombinant DNA technology for the production of relevant microbial protective protein antigens in the appropriate hosts, for vaccine preparation. (2) The use of recombinant DNA techniques for production of live vaccines by introducing the relevant genes into the genome of an adequate vector. (3) The use of naked DNA vaccines, consisting of plasmid DNA into which the relevant genes of the microbial agent has been inserted. (4) The utilization of synthetic peptides which constitute the relevant protective epitopes of pathogens, for eliciting protective immune response towards the disease-causing organism.

#### **1.3.2.2. Subunit vaccines**

Subunit vaccines are defined as those containing one or more pure or semi purified antigens. The foundation for the development of subunit vaccines is the

understanding of the pathogenesis of the pathogen and the proteins, glycoproteins, or carbohydrates involved in inducing protective immunity. Thus, it is critical to identify the individual components of a pathogen that are involved in inducing protective immunity (Babiuk *et al.*, 2002).

Subunit vaccines take advantage of the possibility of using only part of the infectious microorganisms to raise a protective immune response, and since subunit vaccines cannot replicate in the host, there is no risk of pathogenicity. The composition of a subunit vaccine can be clearly defined, which is significant advantage in terms of safety considerations and minimization of side effects. However, the price one has to pay for these advantages is significant. In order to elicit vigorous immune response, subunit vaccines often require multiple doses as well as the use of adjuvants and in many cases can not compete with attenuated and inactivated counterparts (Newman, Powell, 1995). Another challenge associated with the design and development comes from the poor stability of antigens. Aqueous single-vile vaccines that can be stored without refrigeration are highly desirable but are difficult to formulate due to physical and chemical instability of the proteins (St Clair *et al.*, 1999).

Subunit vaccines can be produced by employing recombinant DNA technology or by purifying a specific component from conventionally produced vaccines. Based on this subunit vaccines are classified as recombinant or non -recombinant. Further more, subunit vaccines can be based on peptides, viral surface proteins or bacterial polysaccharides, that have been shown to contain protective epitopes (Hansson *et al.*, 2000; Liljeqvist, Stahl, 1999).

#### **a. Protein based subunit vaccines**

Both humoral and cellular arms of the immune system recognize and react with specific regions of a pathogen due to epitopes recognized by the neutralizing antibodies that are usually found in just one or few proteins present on the surface of the pathogenic organisms. Isolation of genes encoding such epitope-carrying protein immunogens and their expression in heterologous hosts has lead to the design of vaccines based on subunits of the pathogen, namely, protein components that lead to protective effect.

The subunits of pathogenic organisms, such as bacterial polysaccharides, viral surface proteins and detoxified toxins, can be safely used as vaccines or vaccine components. Such protective proteins have, in some cases, been isolated from the

organism, either secreted, as in the case of toxins, or extracted and isolated from the organism after its disruption. This approach is associated with the risk that the extraction method or detoxifying method does not give a pure product. The production of such vaccines also require the large scale production of pathogenic organisms (Hansson *et al.*, 2000; Liljeqvist, Stahl, 1999). Alternatively, such proteins can be synthesized by recombinant DNA technology where the recombinant protein presents an immunogenic part of the pathogen to the immune system and avoids using the whole pathogen (Brown, 1992). Single proteins can be easily produced in various hosts, with myriad possibilities to purpose-design the protein product and also the production process (Koths, 1995).

In 1986, the first recombinant subunit vaccine, the Hepatitis B surface vaccine, was licensed. The vaccine is based on recombinant yeast-derived hepatitis B surface antigen (HbsAg) adsorbed to aluminum adjuvant (Ellis, 1996). The enthusiasm was tempered when it began to appear that hepatitis B may have been a special case where such vaccines might prove both immunogenic and effective for prevention of diseases. For example, FMDV VP1 can be expressed in *E.coli* to levels where up to 17% of the proteins produced in bacteria is VP1, however, the immunogenicity of this protein is less effective than the native protein (Kleid *et al.*, 1981).

Potential challenges in the development of subunit vaccines are that they are poorly immunogenic and have short in vivo half-lives. Another difficulty with subunit vaccines is that they often only elicit strain-specific protection, so, to evoke full protection to a disease caused by several related strains, combinations of immunogens from different strains might be needed. Due to the low immunogenicity of recombinant proteins it is necessary to use immunological adjuvants, but these adjuvants are sometimes ineffective. Thus, to increase the effectiveness of such vaccines it is proposed to use the molecular biology methods for endowing built-in adjuvanticity. These approaches include particulate vaccine presentation systems such as virus-like particles, core-like particles, and other particulate proteins.

#### **b. Peptide based subunit vaccines**

The development of vaccine strategies has seen a move from whole cell organism to recombinant proteins, and further to the epitope. Townsend *et al.* (1985) were the first to report that short peptides could be substituted for whole viral antigen to give T-cell activation (Townsend *et al.*, 1985). Since that time a new trend that has emerged in vaccine development is the quest to re-construct complexity and design vaccines

that contain multiple epitopes or peptides that might induce immunity against multiple antigenic targets, multiple strain variants, and even multiple pathogens (Suhrbier, 1997).

The main benefit of the peptide-based vaccine approach is the ability to produce a peptide antigen which is molecularly defined and pure, and can be characterized with respect to its antigenicity and immunogenicity. This is highly beneficial in terms of safety, effective immune response and large scale production. Not only do these vaccines have increased safety, but there is also lowered antigenic competition between irrelevant proteins, and the ability to differentiate vaccinated animals from infected animals (Babiuk *et al.*, 2002). Additionally, there are certain populations who are at greater risk when considering vaccination with attenuated viruses, namely immunocompromised patients and pregnant woman (Englund *et al.*, 1998). Thus, these peptide vaccines offer a safety advantage in these populations as they are non-replicating. Vaccines contain multiple antibody epitopes that could generate an antibody response to multiple serotypes would clearly be of some value against pathogens such as *Streptococcus*, influenza or AHSV where different serotypes cause disease. Moreover, the development of peptide-based vaccines does not require a reliance on the cold-chain for storage, and this is an important aspect considering mass population vaccine programs in developing countries.

One of the great impediments to the peptide approach is the poor immunogenicity of peptides. This is partially overcome by adding both B and T cell epitopes to the peptide or presenting the peptide in the context of a peptide presentation system. A second problem with peptide vaccines is the elasticity of most pathogens, where quasi-species or mutants can rapidly develop that circumvent the immune response directed at a single epitope. Finally, due to the outbred nature of human and animal populations, the specificity of peptide binding to a specific MHC molecule would preclude mass immunization. These last two problems are partially being overcome by the development of “string-of-beads” vaccines containing multiple epitopes (Whitton *et al.*, 1993) which would be effective in various sections of the population, as well as inducing all arms of the immune response. A case in point is a multiple epitope vaccine against HIV-1 that incorporates three neutralizing epitopes (GPGRAFY, RILAVERYLKD and ELDKWA) of HIV-1 gp160. This vaccine can induce high levels of antibodies to both neutralizing epitopes and ELDKWA RILAVERYLKD, while the GPGRAFY epitope appears to have weak immunogenicity. A gp140 subunit

vaccine can induce only weak-epitope-specific antibody response to these three epitopes (Lu *et al.*, 2000).

Two fundamental immunological restrictions are imposed on epitope vaccines. Firstly, most antibody epitopes are not linear and are comprised of a complex three-dimensional array of scattered residues (Laver *et al.*, 1990; Van Regenmortel, 2001) and are therefore not readily represented by a linear amino acid sequence. This problem can theoretically be overcome by conjugating the peptide to a carrier protein because the surface of the carrier induces a conformation that is better recognized by antibodies (Van Regenmortel, 2001). Secondly, vaccinees who have been exposed to some of the epitopes in a multiple epitope vaccine may not respond well to other epitopes in the vaccine because of the 'original antigenic sin' phenomenon, which results in the boosting of pre-existing B cell responses at the expense of priming naïve B cells specific for the epitopes (Etlinger, 1992; Haynes *et al.*, 1995).

### **C. Synthetic peptides**

Both T and some of the less conformation-dependant B-cell epitopes (linear epitopes) can be mimicked by synthetic peptides. These peptides should be able to elicit an immune response, suggesting that synthetic peptides might be suitable as peptide vaccines (Ertl, Xiang, 1996a; Ertl, Xiang, 1996b).

The major disadvantage of peptide vaccines is their low immunogenicity, especially in outbreed populations, and the monospecificity of the induced immune response, allowing mutating pathogens to escape from immune recognition. Peptides are poor candidates for antiviral vaccines that target large groups of a population; furthermore, the high mutation rate of most viruses precludes vaccines that induce monospecific responses (Bangham, Phillips, 1997). Viral domains prone to neutralization behave as mutational hot spots, especially in RNA viruses (Kinnunen *et al.*, 1990; Tanaka *et al.*, 1991). Based on this it would seem unrealistic to try to develop peptide vaccines against viruses, but if peptide vaccines were based on parts of the viral particle that are not prone to mutation but are vital for its survival, it may remain theoretically possible that full protection could be achieved. Fiers *et al.* (2004) used this approach to design a "universal" human influenza A vaccine based on the external domain of the transmembrane viral M2-protein (M2e). This 24 amino acid peptide is weakly immunogenic but highly conserved and when presented on an appropriate carrier induces a high titer antibody response that in mice effectively protects against a potentially lethal influenza infection (Fiers *et al.*, 2004).

Up to very recently one of the main impediments to the development of synthetic peptide vaccine has been the length of chemically synthesized polypeptides. If the peptide sequence used to represent the epitope is too short it contains insufficient information to fold into the correct shape necessary to mimic conformational-dependant epitopes (Jackson *et al.*, 2000; Van Regenmortel, 2001). In recent years, this problem has been overcome by synthesis techniques which allow the routine production of polypeptides of up to 130 residues, in milligram quantities (Demotz *et al.*, 2001; Lopez *et al.*, 2001).

In 1994 the first study reporting the complete protection against a natural infection by synthetic peptides in the target animal was published by Langeveld *et al.* (1994). Dogs injected with a synthetic peptide vaccine corresponding to an antigenic site on the viral protein VP2 of canine parvovirus were protected against challenge with virulent canine parvovirus (Langeveld *et al.*, 1994). Equally positive results were obtained for synthetic peptide vaccines against respiratory syncytial virus (Bastien *et al.*, 1999) Measles (El Kasmi *et al.*, 2000), Foot-and-Mouth Disease (Wang *et al.*, 2002) and *Yersinia pestis* (Sabhnani *et al.*, 2003).

Chemical synthesis may remain less attractive than recombinant technologies when one considers glycosylation, oligomerization, size of polypeptides and cost of the final products. However, synthetic peptide vaccines have several advantages over other vaccines: they are safe and easy to produce. This approach totally precludes the risk involved in handling and spreading of infectious agents that is associated with the production of conventional vaccines and also ensures the absence of contaminating biological agents in the final product. They can be designed to induce well-defined immune responses; and they can be synthesized with high reproducibility between batches and purity in large quantities (Demotz *et al.*, 2001; Langeveld *et al.*, 1994; Vitiello *et al.*, 1995).

#### **d. Particulate vaccine presentation systems**

Recombinant DNA technology has allowed for the identification, cloning and production of subunit vaccines. However, although these antigens offer advantages in the selection of epitopes and safety, many such highly purified antigens are non-immunogenic or weakly immunogenic. In many cases the use of potent adjuvants are required to elicit a vigorous immune response, but many such adjuvants have adverse reactions. Emphasis should be placed upon utilizing delivery systems to

remedy the limitations of existing immunization regimens (Eldridge *et al.*, 1993). By inserting foreign amino acid sequences into a vaccine presentation system by recombinant DNA techniques, the intrinsic immunogenic advantages of presentation system can be exploited for a whole variety of antigenic determinants (Ulrich *et al.*, 1998). It is also important to take into account that the heterogeneous or particulate nature of antigens increases the likelihood of phagocytosis. Small particles are actively taken up by macrophages to generate primary antibody response. Larger particles, which cannot be phagocytosized, creates a long-term depot effect and thus stimulate strong secondary antibody response (St Clair *et al.*, 1999). Another immunological advantage of particulate structures is their ability to elicit CTL responses (Sedlik *et al.*, 1997). Most conventional vaccines fail to activate a CTL response and thus are ineffective against virally infected cells (Tabata, Ikada, 1988).

In attempts to increase the immunogenicity of antigens, a number of antigen presentation systems have been developed. Existing vaccine presentation systems can be roughly categorized as particulate vaccine carriers, live vaccine vehicles and encoded vaccines (Griffiths *et al.*, 1993). For the purpose of this study, particulate vaccine carriers will be discussed in more detail as presentation system, particularly in the context of the use of AHSV proteins as vaccine carriers.

### **1.3.2.3. Polynucleotide vaccines**

Nucleic acid vaccines constitute a new class of recombinant subunit vaccine, consisting of, for example, plasmid DNA with the gene encoding the antigen of interest under control of a strong mammalian viral promoter. Inoculation of vectors expressing a foreign protein under control of a suitable promoter into the muscle or skin of an animal causes uptake of polynucleotides into cells close to the injection site. In the case of DNA it is subsequently transcribed and translated, causing expression of the vector-encoded protein in the vaccinee (Williams *et al.*, 1991; Wolf *et al.*, 1990).

The immunization of animals, as described above, with naked nucleic acid encoding antigen under the control of a variety of gene regulatory elements has been described in the literature as “polynucleotide immunization”, “DNA-based vaccination”, “somatic transgene vaccination”, “genetic vaccination”, and “nucleic acid vaccination” (Gerloni *et al.*, 1997a; Gerloni *et al.*, 1997b; Robinson *et al.*, 1997). Regardless of terminology, most researchers have settled on “DNA-based” or simply “DNA vaccines” to describe this method of immunization. This form of immunization



was first described in 1992 (Tang *et al.*, 1992) and since that time detractors have described polynucleotide vaccination as “biological cold fusion” (Fomsgaard, 1995), whereas proponents have termed this form of immunization as the “third revolution in vaccinology” (Waine, McManus, 1995).

Genetic vaccines have a number of advantages over traditional vaccines. Firstly, these vaccines are relatively easy to construct and produce. The vaccine acts as a bioreactor, and thus there is no need for downstream processing or formulation of the vaccine. Since adjuvants are not needed, there are none of the adjuvant-associated problems (Babiuk *et al.*, 2002; Bangham, Phillips, 1997; Dertzbaugh, 1998; Ertl, Xiang, 1996b; Hansson *et al.*, 2000). Secondly, antigens are synthesized, post-translationally modified and presented to the immune system of the host in a similar way to that which occurs during natural infection. The protein is processed similarly to an antigen synthesized by an infectious virus, resulting in presentation of antigenic fragments in association with MHC class I determinants which in turn cause activation of cytolytic T cells (Gurunathan *et al.*, 2000c; Ulmer *et al.*, 1993). Genetic immunization in addition results in stimulation of T helper cells and B cells and, in consequence, in protection to challenge (Babiuk *et al.*, 2002; Sedegah *et al.*, 1994; Xiang *et al.*, 1994). Thirdly, although the immune response to genetic vaccines is weak compared with that induced by traditional vaccine, it is exceptionally long lasting (Davis, Whalen, 1995). Fourthly, genetic vaccines are extremely stable and their resistance to heat is highly advantageous when used in developing countries where cold chains are difficult to maintain (Ertl, Xiang, 1996b; Gurunathan *et al.*, 2000a). Fifthly, genetic vaccines can not revert to virulence, making it much safer than conventional vaccines (Bangham, Phillips, 1997; Ertl, Xiang, 1996b). The fifth factor is that these vaccines are highly reproducible and well defined (Bangham, Phillips, 1997), which is of great interest when licensing a vaccine for commercial use. Lastly, these vaccines can induce immune responses in neonates even in the presence of passive antibody and will allow for the vaccination of individuals at a very early age and ensure that they are fully protected at the time passive antibodies decay (Lewis *et al.*, 1999; Van Drunen Littel-van den Hurk *et al.*, 1999).

Nucleotide vaccines can be categorized as DNA vaccines or RNA vaccines.

#### **a. DNA vaccines**

The historical basis for DNA vaccines rests on the observation that direct *in vitro* and *in vivo* gene transfer of recombinant DNA by a variety of techniques resulted in

expression of protein. These approaches include retroviral gene transfer, using formulations of DNA with liposome or proteoliposomes (Kaneda *et al.*, 1989; Mannino, Gould-Fogerite, 1988; Nicolau *et al.*, 1993), calcium phosphate-co precipitated DNA (Benvenisty, Reshef, 1986) and polylysine-glycoprotein carrier complex (Wu, Wu, 1988).

DNA vaccines consist of plasmid DNA expression vectors of *E.coli* origin, which code the antigen or antigens of interest under the control of strong viral promoters recognized by the mammalian host. When the plasmid DNA is administered to an animal the antigen is expressed *in situ* leading to an antigen-specific immunity (Davis, 1997).

DNA vectors that express viral genes have been shown to elicit strong CTL responses and to protect mice and chickens against infection with influenza-A virus (Robinson *et al.*, 1993; Ulmer *et al.*, 1993). Swine inoculated with a DNA vaccine encoding the FMDV genes produced detectable antiviral immune responses (Beard *et al.*, 1999). A clinical trial with DNA coding for hepatitis B surface antigen generated protective levels of antibodies to the antigen and this vaccine also generated CD8 cytotoxic lymphocytes (Rottinghaus *et al.*, 2003). To date only DNA vaccines against hepatitis B and malaria (Wang *et al.*, 1998) have induced immune responses thought to be protective in humans.

A number of safety concerns need to be addressed before considering the development of a DNA vaccine. The most obvious concern is the integration of the vaccine DNA into the host cell genome, causing transformation of the cell and thus cancer, an especially negative prospect for childhood vaccines (Bangham, Phillips, 1997; Dertzbaugh, 1998; Poland *et al.*, 2002). To date, there has been no clear evidence that plasmids integrate, yet neither has this possibility been eliminated (Gurunathan *et al.*, 2000a; Liljeqvist, Stahl, 1999). Another concern is that an immune response could potentially be activated against transfected cells which could lead to the development of autoimmune disease. This concern arises from the immune stimulatory activity of CpG motifs in the plasmid backbone. Bacterial DNA is able to induce the production of anti-double-stranded-DNA auto antibodies in normal mice and accelerate the development of autoimmune disease in lupus-prone animals (Gilkeson *et al.*, 1993). It is also theorized that DNA vaccines can induce tolerance rather than immunity, especially in infants and young children. Newborns exposed to foreign antigens are at risk of developing tolerance rather than immunity (Silverstein,

Segal, 1975) because the protein encoded by a DNA vaccine is produced endogenously and expressed in the context of self-MHC, the potential for the neonatal immune system to recognize it as “self”, resulting in tolerance, rather than immunity.

#### **b. RNA vaccines**

One of the main drawbacks of DNA vaccines is the potential integration of plasmid DNA into the vaccinee’s genome. This risk could be minimized by using RNA instead of DNA in nucleotide vaccines. This approach has been investigated but to a far lesser extent than DNA because of some inherent problems associated with the use of RNA. RNA expression is short-lived and thus less effective in inducing an immune response. Due to low stability of mRNA, preparation and administration of RNA vaccines are not as simple as for DNA (Hansson *et al.*, 2000; Liljeqvist, Stahl, 1999; Stover, 1994).

This approach has the potential to be successful as is demonstrated by Martinon *et al.* (1993) who showed that mRNA encoding the nucleoprotein of influenza virus can efficiently prime virus-specific CTL responses *in vivo* when administered encapsulated in liposomes (Martinon *et al.*, 1993).

### **1.4. AHSV AND BTV PARTICULATE STRUCTURES AS VACCINE DELIVERY SYSTEMS**

The immunogenicity of antigenic peptides can be considerably enhanced by presenting them as multiple copies on the surface of a carrier protein. Because of this a number of systems have been developed in which epitopes are genetically fused to a self-assembling macromolecule.

#### **1.4.1. NS1 Protein**

Gould *et al.* (1994) first suggested the insertion of foreign epitopes into antigenic sites to produce large amounts of epitope incorporated on the surface of the NS1 structure (Gould *et al.*, 1994). This theory is based on the finding by Eaton *et al.* (1988) that the epitopes bound by a panel of monoclonal antibodies are on the surface of the NS1 tubule (Eaton *et al.*, 1988). The epitope of interest can be linked to the self-assembling carrier NS1 protein, which can assemble into predictable multimeric structures and present the inserted foreign epitope to the immune system (Ghosh *et al.*, 2002a; Ghosh *et al.*, 2002b; Ghosh *et al.*, 2002c).

The use of NS1 as a carrier protein is associated with many advantages inherent to the protein such as: (1) high levels of expression, up to 300mg/l culture in Sf cells (25% of proteins synthesized in infected Sf cells) (Urakawa, Roy, 1988); (2) relative ease of purification (Mikhailov *et al.*, 1996); (3) acts as an adjuvant due to the size of the particle. Ghosh *et al* (2002) demonstrated that mice immunized with tubules in the absence of adjuvants generated a higher titer of antibodies than when using standard adjuvants protocols (Ghosh *et al.*, 2002a; Ghosh *et al.*, 2002b).

Mikhailov *et al* (1996) was the first group to investigate the potential of NS1 tubules as epitope carriers. Foreign sequences ranging from 44 to 116 aa in length were inserted at the C-terminus of BTV-10 NS1. The foreign sequences inserted were a 44 sequence from *Clostridium difficile* toxin, 48 aa of the hepatitis B virus preS2 region, and the whole of the bovine leukemia virus p15 protein. All of the baculovirus expressed recombinant proteins still formed tubules and were highly immunogenic. The group also demonstrated that co-infection of Sf cells with the three different recombinant baculoviruses lead to the formation of chimeric NS1 tubules that contained the corresponding three epitopes. This observation opens the way for using recombinant NS1 tubules as carriers for the delivery of multiple epitopes (Mikhailov *et al.*, 1996).

In two recent papers, both published by Ghosh *et al* (2002), the ability of chimeric BTV NS1 tubules to induce protective cytotoxic T lymphocyte (CTL) responses were investigated. Chimeric tubules carrying a single CD8<sup>+</sup> T cell epitope from the lymphocytic choriomeningitis virus (LCMV) nucleoprotein was constructed and were recognized by MHC class I restricted T cell hybridoma *in vitro* and also induced *in vivo* strong CD8<sup>+</sup> class I-restricted CTL responses in immunized mice (Ghosh *et al.*, 2002b). Further, the immunized mice were protected when challenged with a lethal dose of LCMV. To test the hypothesis that more than one epitope can be used to generate chimeric tubules which in turn will induce immune responses against each type of epitope, tubules were constructed that contained two HLA-A2 restricted human melanoma epitopes in tandem. These tubules were capable of inducing CTL responses against both epitopes simultaneously *in vivo* in HLA-A2 transgenic mice (Ghosh *et al.*, 2002c).

In a similar study with BTV NS1 tubules Ghosh *et al* (2002) assessed the extent of response induced following tubule-based immunization by inserting two different immunodominant foreign peptides at the C-terminus of NS1. The two recombinant

NS1 constructs that contained a peptide of *foot and mouth disease virus* (FMDV) (aa135 – 144 of VP1) and a peptide of influenza A virus (aa 186-205 of HA), respectively, assembled into tubules. The tubules carrying the FMDV epitope induced specific anti FMDV antibody response, which protected 60% of immunized mice. This demonstrates very clearly that NS1 tubules that contain a single-copy FMDV epitope could protect immunized mice against virulent FMDV challenge. The chimeric tubules containing HA epitopes induced specific CD4<sup>+</sup> T helper cells in response to influenza A virus. From this it can be concluded that BTV-derived chimeric tubular structures carrying a broad spectrum of immunogenic epitopes can be highly effective against pathogens which require both humoral and cellular responses to overcome infections (Ghosh *et al.*, 2002a).

Based on the ability of BTV NS1 tubules to elicit cellular immune response against displayed immunogenic epitope inserts, this presentation system was explored as vector for priming an HIV-1-specific CD8<sup>+</sup> T-cell response. The chimeric BTV NS1 protein acted as a carrier for the HIVA immunogen, which consists of the HIV-1 clade A gag protein and a string of epitopes recognized by human, monkey and mouse CD8<sup>+</sup> T cells. The HIVA protein is unable to induce T-cell responses efficiently due to its instability and rapid degradation after synthesis. Attachment of the 527-amino-acid-long immunogen HIVA to the C terminus of NS1 caused only minor disturbances of the tubular structure and was able to induce long term (at least 9 months) CD8<sup>+</sup> T-cell-mediated immune responses and afforded the vaccinees partial protection against virus challenge (Larke *et al.*, 2005).

The above studies showed that foreign epitopes attached to the C terminus of BTV NS1 were displayed on the tubule surface without abrogating tubular structure. The immunogenic epitopes displayed on the surface of the tubules were efficiently taken up by professional antigen-presenting cells and were entered the MHC class I pathway. The NS1 tubules carrying the immunogenic epitopes induced protective humoral or cell-mediated responses in the absence of any adjuvant (Ghosh *et al.*, 2002a; Ghosh *et al.*, 2002b; Ghosh *et al.*, 2002c; Monastyrskaya *et al.*, 1995). From these results we can conclude that chimeric NS1 tubules can be highly immunogenic and has great potential for pathogens such as human immunodeficiency virus type 1 (HIV-1) that do not elicit broadly neutralizing antibodies and where the focus of vaccine development are shifting to the stimulation of T-cell mediated immunity (Burton *et al.*, 2004; McMichael, Hanke, 2002).

Similar studies with AHSV NS1 do not exist and it would be of great interest to investigate and compare the potential of AHSV tubules as a peptide presentation system. This study will initiate an investigation of such a nature and will also explore different sites on the AHSV NS1 protein as regions for epitope presentation.

#### **1.4.2. Major Core Protein VP7**

African Horse Sickness virus VP7 is the major serogroup-specific antigen and is located on the surface of the virus core (Chuma *et al.*, 1992; Huismans, Van Dijk, 1990; Oldfield *et al.*, 1990). This highly hydrophobic protein forms trimers (Kowalik *et al.*, 1990; Roy, 1992a), which self-assembles into large, flat, hexagonal crystals (Burroughs *et al.*, 1994; Chuma *et al.*, 1992). This crystal forming ability is unique to AHSV VP7 as BTV VP7 is completely soluble (Oldfield *et al.*, 1990). The AHSV crystals have been demonstrated to be protective against lethal heterologous serotype challenge in a mouse model, but protection was not antibody-mediated. It has been suggested that cell-mediated immunity may play a role in the activation of the observed protective response (Wade-Evans *et al.*, 1997; Wade-Evans *et al.*, 1998). Based on these result and other factors such as self-primed crystal formation even after insertion of foreign sequences at sites 144, 177 and 200 (Maree, 2000; Meyer, 2002; Riley, 2003; Van Rensburg, 2004), relatively high expression levels in recombinant baculovirus–infected cells (Chuma *et al.*, 1992; Maree *et al.*, 1998a; Maree *et al.*, 1998b), and ease of purification an investigation have been launched to evaluate the use of AHSV VP7 as a subunit vaccine system.

As yet no one has been able to prove the effective immune presentation of a peptide on AHSV VP7. It must be emphasized that the lack of response might not indicate that VP7 is a poor presentation system, but in all of these studies emphasis was placed on measuring the humoral immune response that is not necessarily an indication of *in vivo* protection (Meyer, 2002; Riley, 2003).

#### **1.4.3. Virus-Like Particles**

Virus-like particle are generated by spontaneous self-assembly of heterologously expressed viral structural proteins (Ulrich *et al.*, 1998). The enhancement of immunogenicity by VLPs is in part due to the particulate nature of the immunogen, the fact that VLPs often present conformational epitopes to the immune system in a manner that mimics the natural agent and the multimeric structure of identical subunits generates a high epitope concentration and the presence of T-helper epitopes. Not only are these particles advantageous in terms of enhanced

immunogenicity but also in terms of safety because VLPs are noninfectious and lack virus DNA/RNA required for replication (Babiuk *et al.*, 2002; Roy, 1996). A possible drawback of VLPs as a capsid-based delivery system is their limited capacity to accommodate foreign sequences due to packaging constraints (Adams *et al.*, 1987; Adler *et al.*, 1998; Belyaev, Roy, 1993; Clarke *et al.*, 1987; French *et al.*, 1990; Le Blois, Roy, 1993; Miyamura *et al.*, 1994). In spite of this limitation, the success of this approach is demonstrated by the vaccine that is currently in use against hepatitis virus infection that contains yeast-expressed viral surface antigen that is able to self-aggregate and to form non-infectious nHBV-like particles (Ellis, 1996).

BTV virus-like particles, consisting of viral proteins VP2, VP3, VP5 and VP7, have been shown to be highly protective and in addition they are effective at eliciting humoral, cell-mediated and mucosal immunities. These VLPs have also been used as a vaccine delivery system for multiple immunogens (Pearson, Roy, 1993; Roy, 1992b; Roy, 1996).

Although the construction of virus like particles has been successful in BTV, it could not be repeated for AHSV (French *et al.*, 1990; Martinez-Torrecuadrada *et al.*, 1996).

#### **1.4.4. Core-Like Particles**

The BTV CLP has a diameter of 70 nm and consists of VP3 and VP7 trimers that assemble into an icosahedral shape (Grimes *et al.*, 1998). Roy (1996) investigated the use of these particles as a presentation system for foreign peptides by inserting a 15 aa epitope of the Bovine Leukemia Virus (BLV) glycoprotein 51 into VP7 (Roy, 1996). Co-expression of the chimeric VP7 with VP3 leads to the production of intact CLPs that presented the epitope effectively and elicited humoral immune response.

Particles similar to that of BTV have been observed when co-expressing AHSV VP3 and VP7 in an eukaryotic expression system (Burroughs *et al.*, 1994; Le Blois, Roy, 1993; Maree *et al.*, 1998b). Chimeric CLPs were made by Maree (2000) following a strategy similar to that employed by Roy (1996), but has never been tested for immunogenicity. The main problem facing the use of CLPs as a vaccine presentation system is the insolubility of the AHSV VP7 protein that self-assembles into crystals preventing it from associating with co-expressed VP3 to form CLPs. This results in a very low yield of AHSV CLPs and as yet the solubility of VP7 has not been increased successfully (Maree, 2000; Meyer, 2002).

## 1.5. DISEASE PREVENTION AND CONTROL: AHSV

### 1.5.1. Conventional Vaccines

One of the greatest challenges facing development of AHSV vaccines is the fact that any potential vaccine must protect against the multiple serotypes of the virus as vaccination with one serotype does not afford cross-protection against most other serotypes (McIntosh, 1958). For full protection, horses have to be immune to all nine serotypes (Du Plessis *et al.*, 1998). Vaccination against AHSV to avoid new outbreaks is vital as there is no existing treatment for the virus.

The first form of vaccination against AHSV was attempted by Theiler in 1910 when he immunized horses and mules with a mixture of serum recovered from infected animals and virus isolates. This method produced fairly good immunity with a low mortality rate due to immunization and this method was used as prophylaxis from 1910-1933 where after its use was discontinued due to concerns over its efficacy (McIntosh, 1958). In 1934 immunization with neurotropic virus, also called adult mouse brain vaccine (AMBV) was introduced (Alexander, Du Toit, 1934; McIntosh, 1958) and was used in South Africa until 1988 (House, 1998). The use of AMBV was discontinued due to findings that this vaccine caused encephalitis in horses (Shah, 1964) as well as encephalosis and chorioentinitis in laboratory workers exposed to the vaccine (Swanepoel *et al.*, 1992). The other two types of vaccines against AHSV are modified live vaccines (MLV) and inactivated viral vaccines (IVV) (Erasmus, 1965; House, 1998). The vaccine currently in use in South Africa is quadrivalent MLV produced by Onderstepoort Biological Products (OBP) facility in South Africa. This vaccine consist of 2 quadrivalent products, one containing serotypes 1,3, 4, 5 and the other contains serotypes 2, 6, 7, 8 and are administered as two separate injections two weeks apart. The vaccination must be repeated annually for protection and full protection against all serotypes is achieved after 3 – 4 years (Coetzer, Erasmus, 1994; House, 1998)

Although this vaccine has been used with great success, it associated with some disadvantages, real and theoretical, that warrant the development of new and improved vaccines. The foremost concern with this vaccine is the fact that vaccinated horses can not be distinguished from horses naturally infected with the virus (House, 1998). Another real concern is that of immunological interference between the different serotypes in the polyvalent vaccine and this can cause incomplete immunity. A theoretical concern is that reassortment and recombination can take place between



attenuated and virulent strains that could give rise to new strains as well as reversion to virulence (Katz *et al.*, 1990; Oberst *et al.*, 1987; Stone-Marschat *et al.*, 1996).

Based on the constraints associated with existing AHSV vaccines and attenuated vaccines in general it is obvious that the production of a subunit AHSV vaccine is necessary. The development of such a vaccine has been greatly accelerated in recent years by an increased knowledge of the structures and proteins of orbiviruses and it is not impossible that one of these proteins, in particular VP7 and NS1, will form the basis of a new generation recombinant subunit AHSV vaccines.

### **1.5.2. Recombinant Subunit And Peptides Vaccines Based On VP2 Capsid Protein**

VP2 forms part of the virus capsid and together with VP3, VP5 and VP7 forms the two concentric layers around the innermost components of the virus particle (Bremer, 1976; Laviada *et al.*, 1993). Of the outer capsid proteins VP2 is the most variable and exhibits the least conservation between serotypes and serogroups (Fukusho *et al.*, 1987; Iwata *et al.*, 1992a; Iwata *et al.*, 1992b; Vreede, Huismans, 1994) and is the main determinant of serotype specificity (Burrage, Laegreid, 1994; Burrage *et al.*, 1993; Huismans, Erasmus, 1981; Kahlon *et al.*, 1983; Roy *et al.*, 1990b). This is not unexpected if one takes into account that VP2 is the most exposed of the capsid proteins and low identity levels between AHSV and other orbivirus serogroups indicate the evolutionary burden on this protein to continually change. Being located on the outer most surface of the virion, VP2 is subject to constant immunological pressures from the host and alterations within its genome are vital if these orbiviruses are to remain viable within their host system (Williams *et al.*, 1998). The protein functions as the viral haemagglutinin (Cowley, Gorman, 1987; French *et al.*, 1990) facilitating entry into mammalian cells during infection (Mertens *et al.*, 1996) and is also associated with cell adsorption in virus infection as removal of VP2 eliminates binding of the virus to the cell (Eaton, Crameri, 1989).

It is of special interest for the purposes of this study that VP2 is recognized by neutralizing antibodies (Burrage *et al.*, 1993; Martinez-Torrecuadrada, Casal, 1995; Martinez-Torrecuadrada *et al.*, 1999; Vreede, Huismans, 1994). Not only is VP2 recognized by neutralizing antibodies but in BTV it was first established that BTV VP2, isolated from virions and subsequently produced by baculovirus recombinants, induces protective, serotype-specific neutralizing antibodies that protect sheep fully against virulent challenge (Huismans *et al.*, 1987a; Roy *et al.*, 1990b). Baculovirus-

and vaccinia-virus recombinants expressing AHSV VP2 induced protective, humoral, serotype-specific immunity in horses (Martinez-Torrecuadrada *et al.*, 1996; Roy *et al.*, 1996; Stone-Marschat *et al.*, 1996). The fact that VP2 elicits neutralizing antibodies in horses (Roy *et al.*, 1996), rabbits (Martinez-Torrecuadrada *et al.*, 1994) and guinea-pigs (Vreede, Huismans, 1994) suggests that that VP2 may be useful as a subunit vaccine. Unfortunately, studies with baculovirus expressed VP2 from AHSV9 did not induce good protective immune response in horses. This result is due to the insoluble nature of AHSV9 VP2 and in this insoluble form is not ideal for inducing a good protective immune response (Napier, 1999). This is confirmed by previous results with VP2 and VP5 of AHSV5 that indicated that it is the soluble fraction of VP2 which predominantly elicits the neutralizing antibodies (Du Plessis *et al.*, 1998). Soluble recombinantly expressed protein may still effectively present some important epitopes to the immune system, but the insolubility of the baculovirus-expressed VP2 may indicate that the proteins are predominantly incorrectly folded, which may result in the masking of important epitopes. From this it is obvious that the immunogenicity of baculovirus VP2 must be improved before it can be used as an effective subunit vaccine. Based on this a lot of research has been focused on the identification of antigenic regions within VP2 that on their own could elicit neutralizing immune response. The high variability and hydrophobic nature of certain regions of VP2, suggests that these regions are exposed to immunological pressures and may contain epitopes that are serotype specific (Venter *et al.*, 2000). In BTV VP2, neutralization epitopes have been located in positions 328-335 (Gould, Eaton, 1990) and 327-402 (DeMaula *et al.*, 1993). For AHSV4 two neutralization epitopes, 'a' and 'b' between residues 321 and 339 and 377 and 400, respectively (Martinez-Torrecuadrada *et al.*, 2001). In AHSV3 VP2, several antigenic regions were identified between residues 175 and 563 (Bentley *et al.*, 2000) and in AHSV9 VP2 a linear epitope was identified between residues 369 and 403 (Venter *et al.*, 2000). The common location of these neutralizing domains in AHSV and in BTV suggests that these neutralizing regions may be conserved because they are responsible for important functions (Martinez-Torrecuadrada, Casal, 1995). Altogether these results confirm this region to be a major antigenic determinant in AHSV and BTV and an important target for neutralizing antibodies.

## **AIMS OF THIS STUDY**

An investigation of the potential of the using the tubular structures associated with nonstructural protein NS1 of AHSV as a general peptide vaccine delivery system.

This study will be focused on:

1. Determining how NS1 tubules are affected by inserting different peptides and marker protein into an internal site in the NS1 amino acid sequence.
2. Investigation of the potential of NS1 tubular structures to present an immunogenic region of AHSV VP2 and the marker protein eGFP to the murine immune system in a manner that elicits an epitope specific immune response.

## CHAPTER 2

### EXPRESSION OF CHIMERIC AHSV6 NS1 PROTEINS THAT CONTAIN ANTIGENIC AHSV9 VP2 PEPTIDES AND eGFP INSERTS AND THE EFFECT ON TUBULE FORMATION AND IMMUNOGENICITY

#### 2.1 INTRODUCTION

Nonstructural protein NS1 of AHSV, similar to the cognate protein of other orbiviruses, spontaneously polymerizes into macromolecular tubular structures in the cytoplasm of infected cells (Bowne, Ritchie, 1970; Eaton *et al.*, 1987; Huismans, Els, 1979; Nel, Huismans, 1991; Nel *et al.*, 1991). The mainly hydrophobic NS1 protein is characterized by hydrophilic regions that are dispersed over the length of the NS1 protein. In BTV NS1, antigenic epitopes that are specifically recognised by NS1-specific monoclonal antibodies, are located within one of these C-terminal hydrophilic regions (position 474 – 502) and are exposed to the surface of the NS1 tubules that are composed of NS1 protein monomers (Du plessis *et al.*, 1995; Eaton *et al.*, 1988; Monastyrskaya *et al.*, 1995). In theory if a foreign peptide is inserted into such an exposed surface site in the NS1 protein it should be presented to the immune system not only on the surface of the protein monomer, but also in multi-array on the surface of the macromolecular NS1 tubule.

Although no studies presently exist where foreign peptides are inserted into an internal site in the NS1 protein, it has been shown for BTV NS1 that the addition of extra peptides and whole proteins to the carboxy terminus of NS1 has no effect on tubule formation (Ghosh *et al.*, 2002a; Ghosh *et al.*, 2002b; Ghosh *et al.*, 2002c; Mikhailov *et al.*, 1996).

The potential of NS1 tubules as a presentation system for foreign peptides will firstly be evaluated in terms of the ability of chimeric NS1 proteins to spontaneously form stable tubular structures. If the insertion of foreign peptides in an internal site results in the abrogation of tubular structure it would indicate that the peptide is no longer optimally exposed in a predictable manner on the surface of the protein. In this respect it is also important to determine the size limits of peptides that can be inserted into the NS1 protein without destroying tubular structure. The value of the vaccine presentation system will be increased considerably if longer fragments can be inserted. Secondly, the potential of the system will be assessed by the immune response elicited to the fragments inserted into the NS1 protein.

Two of the peptides selected for insertion into the NS1 protein are derived from AHSV9 VP2. This protein is highly variable and is the main determinant of serotype specificity and the neutralization-specific immune response in BTV and AHSV (Burrage *et al.*, 1993; Huismans, Erasmus, 1981; Marshall, Roy, 1990; Martinez-Torrecuadrada, Casal, 1995; Martinez-Torrecuadrada *et al.*, 1994; Martinez-Torrecuadrada *et al.*, 1999; Vreede, Huismans, 1994). Native and baculovirus derived BTV VP2 has been demonstrated to induce neutralizing antibodies and to elicit protection in sheep against virulent challenge (Huismans *et al.*, 1987a; Roy *et al.*, 1990b).

A strong linear AHSV VP2 epitope was identified within a prominent hydrophilic and highly variable domain between amino acids 369 and 403 and it is suggested that that the linear epitope is located partially between amino acids 369-403 (Venter *et al.*, 2000). This area in AHSV9 corresponds well to neutralising domains identified in other AHSV serotypes and BTV (summarized in Table2.1).

**Table 2.1.** Neutralising domains within AHSV and BTV VP2 proteins (Bentley *et al.*, 2000; DeMaula *et al.*, 1993; Gould, Eaton, 1990; Martinez-Torrecuadrada *et al.*, 2001; Venter *et al.*, 2000).

VIRUS	AMINO ACID POSITION OF MAJOR ANTIGENIC SITES	REFERENCE
AHSV9	369 to 403	(Venter <i>et al.</i> , 2000)
AHSV4	321 to 339	(Martinez-Torrecuadrada <i>et al.</i> , 2001)
AHSV4	377 to 400	(Martinez-Torrecuadrada <i>et al.</i> , 2001)
AHSV3	175 to 563	(Bentley <i>et al.</i> , 2000)
BTV10	327 to 402	(DeMaula <i>et al.</i> , 1993)
BTV	328 to 335	(Gould, Eaton, 1990)

Two overlapping regions of AHSV9 VP2, of 40 and 150 amino acids, were selected based on these regions of AHSV and BTV VP2.

**Table 2.2.** Regions of AHSV9 VP2 selected for insertion into AHSV6 NS1.

Insert size	Nucleotide position in the coding region of the VP2 gene	Amino Acid position in the VP2 protein
40 amino acids	1091 to 1212	363 to 404
150 amino acids	805 to 1257	268 to 419

The third peptide selected for insertion into the NS1 protein is the full length enhanced green fluorescent protein (eGFP) (240 aa). Enhanced eGFP is a red-shifted variant of wild-type green fluorescent protein (GFP) (Chalfie *et al.*, 1994; Inouye, Tsuji, 1994; Prasher *et al.*, 1992) which is optimized for brighter fluorescence and higher expression in mammalian cells. The coding GFPmut1 variant of eGFP (Cormack *et al.*, 1996) contains the double-amino acid substitution of Phe-64 to Leu and Ser-65 to Thr (Haas *et al.*, 1996).

## 2.2 MATERIALS AND METHODS

### 2.2.1 Materials

The original pBluescribe and pFastBac plasmid with the coding region of the AHSV6 NS1 gene inserted into the *BamHI* site of the MCS was provided by Dr. F. F. Maree of the department of Genetics, University of Pretoria (Maree, 2000). The cDNA clone of AHSV9 segment 2 (VP2) in plasmid pBS9.2Hyb was obtained from the department of Genetics, University of Pretoria (Napier, 1999). The eGFP gene in the pGEM-t-easy™ vector was obtained from L. Burger of the department of Microbiology, University of Pretoria. All primers were purchased from Invitrogen (Life Technologies). Suppliers of kits, enzymes and other materials are indicated within the methods section. All other general reagents and chemicals were obtained from Merck and Sigma- Aldrich.

### 2.2.2. Nucleotide Sequence Determination of the AHSV6 NS1 gene and Chimeric NS1 constructs

Automated sequencing was used to confirm the sequence authenticity of the existing cloned AHSV6 NS1 gene (Maree, 2000) and also to confirm insert orientation and sequence integrity of chimeric NS1 constructs.

The nucleotide sequences were determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Universal M13 primers (Table 2.3) were

used for forward and reverse priming. Two additional internal NS1-specific primers, NS1-627-F and NS1-627-R, were also used for complete sequencing of the genes (Table 2.3).

**Table 2.3.** Primers used for sequencing of recombinant NS1 genes

PRIMER NAME	NUCLEOTIDE POSITION	PRIMER SEQUENCE
M13F	-	5' TGT AAA ACG ACG GCC AGT 3'
M13R	-	5' CAG GAA ACA GCT ATG AC 3'
NS1-627-F	628 – 641 of AHSV6 NS1 gene	5' GAA GGG CGA TTG CCC GTT TGC 3'
NS1-627-R	628 – 641 of AHSV6 NS1 gene	5' GCA AAC GGG CAA TCG CCC TTC 3'

#### 2.2.2.1. Cycle sequencing

All sequencing was done by automated sequencing and the cycle sequencing reactions were set up with the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems). The reactions were set up in a total volume of 10 ul and consisted of 250-500 ng purified double-stranded plasmid template, 3.2 pmol primer, 2ul BigDye® and 5 x sequencing buffer and was made up to its final volume with Sabax® water. The cycle sequencing reaction was carried out in a Perkin Elmer GeneAmp PCR system 9600 using the BigDye® program. The cycle sequencing products were purified from excess dye terminators and salts by a sodium acetate-EDTA precipitation. The reactions were mixed with 1 ul EDTA (125 mM), 1ul NaAc (3M) and 25 ul absolute Ethanol and incubated for 15 minutes at room temperature. The incubation step was followed by a 15 minute centrifugation step at 1650 g. The supernatant was then discarded, the pellet washed with 35 ul 70% ethanol and the pellet collected by centrifugation step at 16 000 g for 15 minutes.

The precipitated cycle sequencing products were resuspended in Hi-Di™ formamide (Applied Biosystems) before loading on the ABI 3100 automated sequencer (Applied Biosystems). The sequences were analysed by using the ABI PRISM Sequencing Analysis™ and Sequence Navigator™ programs. Electropherograms of all the sequencing reactions of a specific DNA template were imported into Sequence Navigator™ Version 1.0.1 and aligned and checked for ambiguous bases. The

consensus sequences that were obtained from the alignments were used to align with theoretical sequences of the specific DNA template in sequence alignments performed in ClustalX version 1. 8 (Thompson *et al.*, 1997).

### **2.2.3. Hydropathy and Antigenicity Predictions**

Protein sequences were analysed using the ANTHEPROT computer package (Geourjon, Deleage, 1995; Geourjon *et al.*, 1991). This software package employs the Hopp and Woods predictive method (Hopp, Woods, 1981; Hopp, Woods, 1983) and the method described by Welling *et al.* (1985) to draw hydrophilicity and antigenicity plots of proteins, respectively (Welling *et al.*, 1985).

### **2.2.4. Amplification of VP2 and eGFP Inserts and Introduction of Terminal *Xho*I Sites**

#### **2.2.4.1. Polymerase chain reaction**

The cDNA clone of AHSV9 segment 2 (VP2) in plasmid pBS9.2Hyb (Napier, 1999) was used as template for the amplification of the 120 and 450 base pair regions (Table 2.2) and incorporation of *Xho*I sites onto the ends of the amplified regions by polymerase chain reaction (PCR). The eGFP gene in the pGEM-t-easy<sup>TM</sup> vector was obtained from L. Burger of the department of Microbiology, University of Pretoria and was used as template for the amplification of the complete eGFP gene including start codon but excluding stop codon. The primers are summarized in Table 2.4. The regions highlighted in green correspond to sequences complimentary to AHSV9 VP2 or eGFP and the sequences highlighted in yellow show the terminal restriction enzyme site *Xho*I.



**Table 2.4.** A summary of primer sequences used in the amplification of AHSV9 VP2 fragments and the eGFP gene.

PRIMER NAME	FUNCTION	NUCLEOTIDE POSITION	OLIGONUCLEOTIDE SEQUENCE
VP2F	Amplification of a 120 nucleotide region of AHSV9 VP2	1103-1121	5'- CAGCTCGAGCCCTTGAGGTGTTTAA CG-3'
VP2R		1210-1225	5'- CAGCTCGAGCTAAGTTGGAATTCGC- 3'
VP270F	Amplification of a 450 nucleotide region of AHSV9 VP2	817-837	5'- CAGCTCGAGCCGAAGACGAAGAATT GAGTCGG-3'
VP420R		1249-1269	5'- CAGCTCGAGCCACTTGTAGATAAGCT GACGC-3'
eGFP-F	Amplification of the 717 nucleotide eGFP gene without stop codon	1 -15	5' - GTGTCAGCTCGAGCCATGGTGAGCA AGGGC - 3'
eGFP-R		703 - 717	5' - GTGTCAGCTCGAGCCTTGACAGCTC GTC - 3'

The PCR reactions were performed using Takara ExTaq™ DNA polymerase and provided reagents. The reactions were set up in a final volume of 100 µl as follows: a tenth of the final volume 10 x Takara buffer (provided), 0.5 µl of 2.25 mM dNTP mix, 100 pmol of the forward and reverse primer (Table 2.4), 10 ng template DNA, 2.5 units (U) TaKaRa Ex Taq™ polymerase were made up to final volume with ddH<sub>2</sub>O. A negative control was also set up that consisted of a reaction mix identical to the above, but template DNA was replaced with ddH<sub>2</sub>O. The PCR reactions were performed in a GeneAmp PCR system 9600 (Perkin Elmer). A four-stage

amplification program was used to amplify the AHSV9 VP2 fragments and eGFP gene (Table 2.5).

**Table 2.5.** Four-stage program for the amplification of AHSV9 VP2 120 and 450 nucleotide fragments as well as the eGFP gene.

Amplification product	Stage	Step	Temperature	Time	Cycles	
AHSV9 VP2 120 nucleotide fragment	1	Initial denaturation	94°C	2'	1	
	2	Denaturation	94°C	30 sec	5	
		Annealing	50°C	30 sec		
		Elongation	72°C	60 sec		
	3	Denaturation	94°C	30 sec	25	
		Annealing	56°C	30 sec		
		Elongation	72°C	60 sec		
	4	Final Elongation	72°C	7 minutes	1	
	Amplification product	Stage	Step	Temperature	Time	Cycles
	AHSV9 VP2 450 nucleotide fragment	1	Initial denaturation	94°C	2'	1
2		Denaturation	94°C	30 sec	5	
		Annealing	52°C	30 sec		
		Elongation	72°C	60 sec		
3		Denaturation	94°C	30 sec	25	
		Annealing	57°C	30 sec		
		Elongation	72°C	60 sec		
4		Final Elongation	72°C	7 minutes	1	
Amplification product		Stage	Step	Temperature	Time	Cycles
		1	Initial denaturation	94°C	2'	1
	2	Denaturation	94°C	30 sec		

<b>eGFP gene without stop codon</b>		Annealing	50°C	30 sec	5
		Elongation	72°C	60 sec	
	3	Denaturation	94°C	30 sec	25
		Annealing	56°C	30 sec	
		Elongation	72°C	60 sec	
	4	Final Elongation	72°C	7 minutes	1

#### 2.2.4.2. Purification of amplified VP2 and eGFP DNA fragments

Amplified PCR products were purified directly from PCR reactions using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). With the exception of the elution step, the purifications were performed according to manufacturer's instructions. The DNA was eluted in ddH<sub>2</sub>O instead of the provided elution buffer.

#### 2.2.5. Cloning of VP2 Regions and The eGFP Gene into pBS-NS1

##### 2.2.5.1. General

The pBS-S5Hyb vector was obtained from Dr. F. F. Maree (UP). In this vector, a chimeric AHSV6 NS1 gene is inserted into the *Bam*HI site of a pBS transcription vector under the control of the T7 promoter. The gene lacks most of the 5' and 3' non-coding regions, except for the Kozak sequence that flanks the AUG codon. The NS1 gene contains a unique *Xho*I site that corresponds to a hydrophilic domain within the hydrophobic C-terminal region of the NS1 protein (as discussed in section 2.3.2). The inserts were cloned into the pBS-NS1 vector, because the pBluescribe vector lacks a *Xho*I site. For the purpose of cloning the VP2 regions and eGFP gene into NS1, both the pBS-NS1 vector and PCR amplified regions were digested with *Xho*I.

##### 2.2.5.2. Restriction endonuclease digestion of the vector and inserts

The restriction enzyme digestions were performed according to manufacturer's recommendation (Roche Diagnostics). Restriction enzyme digestions were performed in the presence of the appropriate 10 x buffer provided by the manufacturer, made up to 10% of the final reaction volume. The DNA preparations were digested for the appropriate time at the recommended temperature.

##### 2.2.5.3. Analysis of DNA by agarose gel electrophoresis

Digested DNA samples were analysed on horizontal 1% or 2% agarose gels (w/v), depending on the size of the DNA fragment, in the presence of 0.5 ug/ul ethidium bromide and 1x TAE buffer (40mM Tris-HCl, 20mM Na-acetate, 1mM EDTA, pH 8.0). DNA samples were loaded on to gels in the presence of loading buffer (1% bromophenol blue, 1% xylene cyanol, 1xTAE buffer, 50% glycerol) and visualized under UV light.

#### **2.2.5.4. Purification of Digested DNA from Solution**

*Xho*I digested pBS-NS1 vector and PCR products were directly purified from restriction enzyme reactions using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The purifications were performed according to manufacturer's instructions, with the exception of the elution step. The DNA was eluted in ddH<sub>2</sub>O instead of the elution buffer provided in the kit.

#### **2.2.5.5. Dephosphorylation of vector**

The *Xho*I linearised pBS-NS1 was dephosphorylated with shrimp alkaline phosphatase (Roche). Reactions were set up so that the final reaction consisted of 10% dephosphorylation buffer (0.5 M Tris-HCl, 50 mM MgCl<sub>2</sub>, pH 8.5) and 10% phosphatase alkaline. The reaction was incubated at 37°C for 1hour. At the end of the incubation period, the enzyme was heat inactivated at 65°C for 15 minutes and used directly for ligation reactions.

#### **2.2.5.6. Ligation**

The purified PCR fragments and linearised, dephosphorylated vector was ligated for 16 h at 16°C in the presence of one unit T4 DNA ligase and 1x ligation buffer (66 mM Tris-HCl, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 1mM ATP, pH 7.5). The *Xho*I digested PCR fragments were ligated to the dephosphorylated pBS-NS1 vector in a 3:1 molar ratio.

#### **2.2.5.7. Preparation of competent *E.coli***

The CaCl<sub>2</sub>-method (Cohen *et al.*, 1972; Mandell, Higa, 1970) was used to prepare competent *E. coli* (XL1-Blue) cells. A cell culture was prepared by inoculating 250 ml Lauria-Bertani (LB) medium (1% NaCl (w/v), 1% tryptone (w/v), 0.5% yeast (w/v) with 1 ml of an overnight culture and incubated with shaking at 37°C until the cell culture reached logarithmic phase (OD<sub>550</sub> = 0.45–0.50). Cells were collected from growth medium by centrifugation at 5000 rpm for 5 minutes at 4°C. The cell pellet was then resuspended in half the original volume of ice cold 50 mM CaCl<sub>2</sub> and incubated on ice for at least 30 minutes. After incubation the cells were once again collected by

centrifugation and resuspended in 0.05% of the original collection volume of 50 mM ice cold CaCl<sub>2</sub>. The resuspended cells were then incubated on ice for 1 hour before direct use in transformation or flash frozen in liquid nitrogen in the presence of 15% (v/v) sterile glycerol for long-term storage at -70°C.

#### **2.2.5.8. Transformation of competent cells**

Chemically competent XL1-Blue *E. coli* cells were transformed by a heat-shock method (Sambrook *et al.*, 1989). The ligation mixture was combined with 100 µl competent cells and incubated on ice for at least 30 minutes to allow binding of DNA to the cell membranes. The chilled translation mixture was then heat-shocked at 42°C for 90 seconds and then rapidly cooled on ice for 2 minutes to facilitate the uptake of DNA into cells. The heat shocked cells were then allowed to recover at 37°C with shaking in the presence of 1 ml preheated LB-broth (without antibiotics). After this 1 hour recovery period the cells were plated out on LB-agar plates supplemented with tetracycline hydrochloride (12.5 µg/ml) and ampicillin (100 µg/ml). Plates were then incubated at 37°C overnight.

#### **2.2.6. Plasmid DNA Isolation And Purification**

Plasmid DNA was extracted from liquid cultures of *E. coli* cells by the alkaline lysis method (Birnboim, Doly, 1979). Single bacterial colonies were used to inoculate 5 ml LB-broth, supplemented with the appropriate antibiotics, and incubated with shaking at 37°C overnight. Cells from 1.5 ml culture were collected by centrifugation at 13200 rpm for 1 minute and the collected pellets resuspended in 200 µl cold Solution 1 (50 mM glucose; 10 mM EDTA; 25 mM Tris; pH 8.0). After incubation at room temperature for 5 minutes, 200 µl of Solution 2 (0.2 M NaOH; 1% SDS) was added to ensure complete lysis of bacterial cells as well as denaturation of proteins and DNA (genomic and plasmid) and incubated on ice for 5 minutes. Genomic DNA and plasmid DNA were separated by adding 200 µl 3M NaAc (pH 4.8), hence precipitating genomic DNA and high molecular weight RNA, while at the same time reannealing the plasmid DNA. After 30 minutes incubation on ice, the precipitates and cell debris were pelleted by centrifugation at 13200 rpm for 20 minutes at 4°C. The plasmid DNA in the cleared supernatant was ethanol precipitated and collected by centrifugation at 13200 rpm for 1 minute. The pellets were then washed consecutively with 70% and 80% ethanol to remove excess salt and then vacuum dried and resuspended in 50 µl ddH<sub>2</sub>O. To obtain plasmid DNA samples free of

contaminating RNA and protein, plasmid DNA samples were column purified with the High Pure™ Plasmid Purification Kit (Roche Diagnostics).

## 2.2.7. Characterization of Recombinant Plasmids

### 2.2.7.1. Restriction endonuclease digestion

Isolated plasmids were analysed on horizontal 1% agarose gels (w/v) in the presence of 0.5 ug/ul ethidium bromide and 1x TAE buffer (40mM Tris-HCl, 20mM sodium acetate, 1mM EDTA, pH 8.0). Putative recombinant plasmids were first selected based on size (recombinant plasmids seems larger than control on agarose gel). The selected plasmids were then further screened by restriction enzyme digestions with *XhoI* (Roche) and *BamHI* (Roche), respectively. Restriction enzyme digestions were performed as described in section 2.2.5.2. Restriction enzyme products of *XhoI* digestion were analysed on 2% agarose gels and *BamHI* digestions on 1% agarose gels (section 2.2.5.3).

### 2.2.7.2. PCR-based orientation selection

Due to the non-directional cloning strategy it was necessary to determine the orientation in which inserts were cloned into the *XhoI* site of the NS1 gene. PCR was used to determine the orientation of inserts by using an insert-specific forward primer and vector-specific reverse primer that binds downstream (in MCS of pBluescribe) of the gene (Table 2.6). A recombinant gene that contains the insert in the incorrect orientation is not expected to give an amplification product, as the orientation of amplifying primers does not intersect.

5ng template DNA was used to set up a PCR reaction with Promega Taq (Promega). The reaction consisted of 10 ul 10x buffer (provided with Taq enzyme), 6.5 ul 25 mM MgCl<sub>2</sub> (provided with Taq enzyme), 100 pmol of the appropriate forward primer, 100 pmol of the M13R primer and 2.5 U of the polymerase enzyme. The final volume of the reaction was made up to 100 ul with ddH<sub>2</sub>O. Negative controls were performed in parallel with the PCR reactions. The negative controls consisted of reactions identical to above reactions except that template DNA were replaced with ddH<sub>2</sub>O. 10% of PCR reactions were analysed on 1% agarose gels.

**Table 2.6.** Summary of primers used for PCR orientation selection of recombinant NS1 genes.

Insert	Insert-specific forward primer	Vector-specific reverse primer
VP2-40	VP2F	M13R
VP2-150	VP270F	M13R
eGFP gene	eGFP-F	M13R

The PCR reactions were performed in a GeneAmp PCR system 9600 (Perkin Elmer). A three-stage amplification program was used to amplify the fragment between the forward and reverse primer as summarized in Table 2.7.

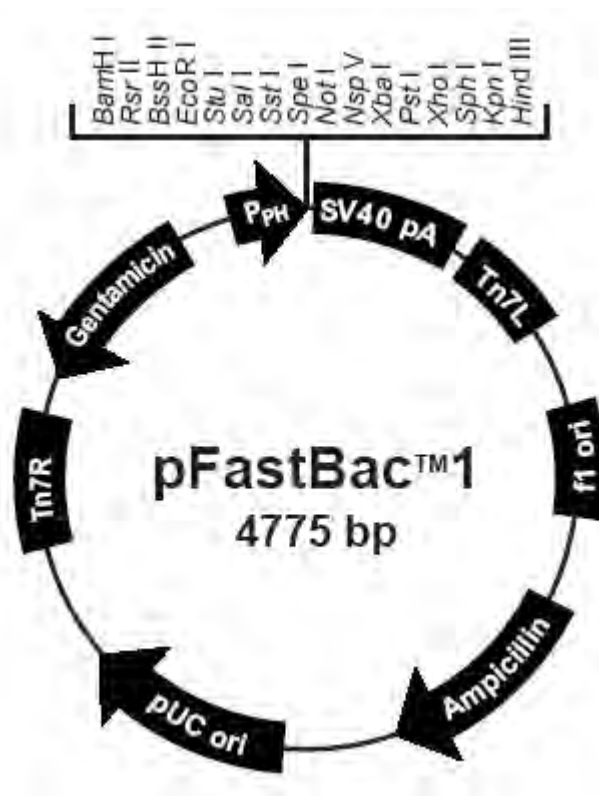
**Table 2.7.** Three-stage Program for the amplification of nucleotide fragments to determine orientation of inserts in the NS1 gene.

Stage	Step	Temperature	Time	Cycles
1	Denaturation	94°C	2'	1
2	Denaturation	94°C	30 sec	30
	Annealing	58°C	30 sec	
	Extension	72°C	1'	
3	Extension	72°C	7'	1

## 2.2.8. Expression Of Recombinant NS1 Proteins with the BAC-TO-BAC® baculovirus expression system

### 2.2.8.1. Construction of a recombinant bacmid transfer vector

The plasmid pFastBac1 (Life Technologies) is the donor plasmid of the BAC-TO-BAC Baculovirus expression system. This plasmid makes it possible to clone foreign genes in *E. coli* and subsequently transfer the recombinant gene into the baculovirus genome by transfer via Tn7 site-specific transposition. A polyhedrin promoter is situated upstream of the pFastBac multiple cloning site and drives the expression of the foreign genes inserted into the multiple cloning site (Figure 2.1).



**Figure 2.1.** Plasmid map of pFastBac1 (Life Technologies)

The recombinant NS1/VP2 -40, NS1/VP2-150 and NS1/eGFP genes were excised from the pBluescribe vectors by *Bam*HI restriction enzyme digestion (as described in 2.2.5.2) and purified from 1% agarose gel using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), as described in section 2.2.4.2. The pFastBac transfer vector (Figure 2.1) was also digested with *Bam*HI (as described in section 2.2.5.2.), the linearised vector purified from solution (as described in section 2.2.5.2.) and the dephosphorylated (as described in section 2.2.5.4). The respective recombinant NS1 genes were then ligated to the *Bam*HI digested, dephosphorylated pFastBac1 vector (as described in section 2.2.5.6). Competent *E. coli* XL1-blue cells were transformed with ligation reactions and plated out on LB agar plates with added ampicillin and tetracycline (as described in 2.2.5.8). Antibiotic resistant colonies were liquid cultured in LB broth in the presence of ampicillin (100 ug/ml) and tetracycline (12.5 ug/ml). Plasmid DNA was extracted from liquid cultures of *E. coli* cells by the alkaline lysis method (section 2.2.6).

### 2.2.8.2. Characterization of recombinant bacmid transfer vectors

Putative recombinant plasmids were selected based on size using electrophoresis on 1% agarose gels (section 2.2.5.3). The recombinant plasmids appear to have an



increased size when compared to controls (pFB-NS1). Selected plasmids were then further screened by restriction enzyme digestion with *Bam*HI (Roche) and *Hind*III (Roche), as described in section 2.2.5.2, to select plasmids that contained the correct recombinant gene and in the correct orientation relative to the polyhedrin promoter.

### 2.2.8.3. Cell culture

*Spodoptera frugiperda* (Sf9) cells, used for baculovirus infection, were obtained from the NERC Institute of Virology, Mansfield Road, Oxford, UK. Cell culture techniques were essentially performed as described in the BAC-TO-BAC® baculovirus expression system manual (GIBCOBRL, Life technologies). The Sf9 cells were grown in confluent monolayers or as suspension cultures at 27°C in Grace's insect medium supplemented with 10% (v/v) fetal calf serum, an antimycotic solution (0.12 mg/ml penicillin, 0.12 mg/ml streptomycin and 0.0325 µg/ml Fungizone) (Highveld Biological) and 10% Pluronic-F68 (Sigma). The density and viability of Sf9 cells were determined by counting cells under a light microscope on a hemacytometer. Before counting the cells were stained with 0.4% trypan blue solution (in 1x PBS) in a 1:1 ratio of cell culture to stain. Only cells that did not take up the trypan blue stain were considered viable and counted to determine cell density.

### 2.2.8.4. Transposition and isolation of bacmid DNA

For the purpose of transposing the recombinant NS1 genes into the bacmid genome (baculovirus shuttle vector) within *E. coli* DH10BAC™ cells (Life Technologies), the cells were made competent by the DMSO method (Chung, Miller, 1988). The cells were grown to early log phase ( $OD_{550} = 0.5$ ) in LB-broth supplemented with kanamycin (50 µg/ml) and tetracycline hydrochloride (12.5 µg/ml). The cells from 100 ml liquid culture were then collected by centrifugation at 4000–5000 rpm for 5 minutes at 4°C and resuspended in 10% of the original culture volume (10 ml) ice-cold TSB (LB-medium supplemented with 10% w/v PEG, 5% v/v DMSO, 10 mM  $MgCl_2$  and 10 mM  $MgSO_4$ ) and then incubated on ice for 10 to 20 minutes after which it is ready for transformation. 200 µl of the competent DH10BAC cells were mixed with between 100 and 200 ng of the donor plasmid (pFastBac-NS1/VP2-40, or -150, or -NS1/eGFP) and incubated on ice for 40 minutes. After the incubation step 0.8 ml TSBG (TSB with 20 mM glucose) was added and the cells incubated with shaking at 37°C for 4 hours before being plated out on LB-agar plates supplemented with tetracycline hydrochloride (12.5 µg/ml), kanamycin (50 µg/ml), gentamycin (7 µg/ml), X-gal (300 µg/ml) and IPTG (40 µg/ml). The plates were then incubated at 37°C for

at least 24 hours to allow for the sufficient growth of colonies and expression of blue/white phenotypes. The bacmid genome within the DH10Bac cells contains *lacZ* gene and if transposition of the Tn7 element into the bacmid genome occurs, the *lacZ* gene is disrupted and recombinant colonies will be blue as compared to the non-recombinant colonies that are white. The white colonies were then used to inoculate 5ml LB-broth, supplemented with tetracycline, gentamycin and kanamycin and incubated at 37°C overnight.

Composite and wild type bacmid DNA were extracted from *E.coli* DH10BAC cell cultures using a modified alkaline lysis method adapted for the extraction of high molecular weight DNA (Lucklow *et al.*, 1993). The cells from 1.5 ml culture were collected by centrifugation at 14000 g for 1 minute and the pellet was then resuspended in 0.3 ml Solution 1 (15 mM Tris- HCl, 10 mM EDTA). The resuspended cells were then lysed by the addition of 0.3 ml Solution II and incubated at room temperature for 5 minutes. Incubation was followed by the addition of 300 ul 3M KAc (pH = 5.5) to the sample and an incubation step on ice for 5–10 minutes to precipitate protein and genomic *E. coli* DNA. The precipitated protein and genomic DNA were collected by centrifugation at 14 000 g for 20 minutes and the cleared supernatant was then transferred to a new eppendorf tube. The bacmid DNA was precipitated out of the collected supernatant by the addition of 0.8 ml absolute isopropanol and incubation on ice for 10 minutes. The Bacmid DNA precipitate was then pelleted by centrifugation at 14 000 g for 15 minutes and the resulting pellet was then washed with 0.5 ml 70% ethanol to remove contaminating salts. After the 70% ethanol wash the pelleted DNA was air dried and resuspended in 40 ul sterile SABAX® water.

#### **2.2.8.5. Production and identification of recombinant baculoviruses**

In preparation of transfection Sf9 cells were seeded at  $1 \times 10^6$  per 35-mm well in 2ml of Grace's medium supplemented with 10% (v/v) fetal calf serum, and antimycotic solution ( 0.12 mg/ml penicillin, 0.12 mg/ml streptomycin and 0.0325 ug/ml Fungizone) (Highveld Biological). After seeding, the cells were allowed to attach at 27°C for at least 1 hour. While cells were attaching mixtures of isolated bacmid DNA and the lipid complex reagent, CellFECTIN™ (Invitrogen) were prepared as follows: for each transfection 5 ul of the Bacmid DNA was diluted with 100 ul Grace's medium without antibiotics and fetal calf serum and in duplicate a separate dilution of 6 ul CellFECTIN reagent in 100 ul unsupplemented Grace's medium was made for each transfection. The two dilutions were then combined, mixed and incubated for 45

minutes at room temperature. During this incubation period the attached cells were washed once with 2ml of unsupplemented Grace's medium to remove all traces of fetal calf serum and antimycotics. At the end of the 45 minute incubation period, an additional 800 ul clean Grace's medium was added to each individual tube. Wash media was then aspirated from Sf9 cells and overlaid with 1ml of the diluted lipid-DNA complexes. The cells were then incubated with lipid- DNA complex dilution for 16 hours at 27°C before removing the transfection mixture and replacing it with supplemented Grace's medium. Cells were also transfected with wild- type viral DNA isolated from blue colonies generated during the transposition step .The cells were then incubated for an additional 72 hours at 27°C after which the virus-containing medium was collected and stored at 4°C for future use.

The transfections were assayed for recombinant protein expression before large-scale virus amplification. 35-mm wells were infected with 200 ul of the virus-containing medium collected from transfections. At 72 hours post infection cells were harvested and proteins from infected cells resolved by SDS-PAGE (section 2.2.9).

#### **2.2.8.6. Preparation of viral stock**

Viral stocks were prepared from transfection stocks (section 2.2.8.5) that were assayed for recombinant or wild type viral expression. 75 cm<sup>3</sup> tissue culture flasks were seeded at a density of  $1 \times 10^7$  Sf9 cells per flask. The cells were allowed to attach before replacing the medium with 10 ml supplemented Grace's medium. Each flask was then infected with 200 ul of the transfection medium and incubated at 27°C for 72 hours. The medium containing the amplified virus was then aspirated from the cells and the medium cleared from Sf9 cells by centrifugation at 1500 rpm for 5 minutes. The cleared supernatant was then filter sterilized with 0.2 um pore acetate filters (Sigma) and stored at 4°C for future use.

#### **2.2.8.7. Expression of recombinant NS1 proteins**

Small- and large- scale infections of Sf9 cells were performed to obtain different levels of protein expression. Small- scale protein expression was performed in either 24- or 6-well plates at cell densities of  $0.3 \times 10^6$  and  $1 \times 10^6$  cells per well, respectively. For 24-well infections the cells were seeded in a maximum volume of 500 ul Grace's medium supplemented with fetal bovine serum, antibiotics and Fungizone. After cell attachment the medium was replaced with 350 ul supplemented Grace's medium, the cells infected with 150 ul of the baculovirus stock, and cells

were then incubated at 27°C for 72 hours before cell harvesting. Following the same approach, cells seeded in 6 wells were first allowed to attach to the surface of the well before the medium was replaced with 1.8 ml of fully supplemented Grace's medium and infected with 200 ul of the baculovirus stocks. The infected cells were then incubated at 27°C for 72 hours to allow protein expression. The same infection protocol was followed for both recombinant baculoviruses as well as wild type baculoviruses. For each set of baculovirus infections mock infections were also performed where virus inoculums were replaced with sterile, supplemented Grace's medium hence allowing optimal expression of cellular Sf9 cells without baculovirus infection.

Some protocols such as sucrose gradient density centrifugation (section 2.2.11), storage stability studies (section 2.2.12) and animal trials (section 2.2.15) require high concentrations of baculovirus-expressed proteins and the required levels of protein expression is achieved by large-scale Sf9 cell infection. For large-scale protein expression,  $1 \times 10^7$  Sf9 cells in fully supplemented Grace's medium were seeded in 75 cm<sup>3</sup> tissue culture flasks and left at 27°C for at least 1 hour to immobilize on plastic surface. Once cells were attached sufficiently the medium was replaced with 7 ml supplemented Grace's medium and infected with 500 – 1000 ul virus stock (M.O.I. of 5- 10 pfu/cell) per flask. The infections were then incubated at 27°C for 2-5 hours before adding an additional 8 ml of supplemented Grace's medium to each flask. The two-step addition of medium to virus infections is a form of synchronized virus infections that optimizes the probability of virus attachment to individual cells. The large-scale infections were harvested at 72 hours post infection.

#### **2.2.8.8. Harvesting of cells**

Cells from 24-wells and 6-wells were harvested by aspirating the medium from cells and then mechanically detaching cells from the surface in the presence of 500 ul or 1000 ul 1 x PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, pH = 7.4), respectively. After collecting the cells by centrifugation at 3000 rpm for 5 minutes the cells were resuspended in 30–50 ul 0.15 STE buffer (10 mM Tris, 1 mM EDTA, 150 mM NaCl, pH = 7.4). A similar approach was followed to harvest cells from 75 cm<sup>3</sup> flasks, i.e. cells were mechanically removed from the flask surface in the presence of medium. The cells were then collected from the medium by centrifugation at 3000 rpm for 5 minutes and the collected cells were then washed with 10 ml 1 x PBS. The washed cells were then resuspended in 1ml 0.15 M STE buffer.

### **2.2.9. SDS Polyacrylamide Gel Electrophoresis (PAGE)**

Before electrophoretic analysis, protein samples (in 0.15 M STE) were treated with equal amounts of 2x protein solvent buffer (125 mM Tris-HCl pH = 6.8., 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue) and then denatured by heating the samples to 95°C for 5 minutes. The protein samples were then resolved on 10% or 12% SDS-PAGE gels (Laemmli, 1970). The 0.75 mm thick separating gels contained 0.375 M Tris-HCl, pH8.8 and 0.1% SDS and the 5% stacking gel contained 0.125 M Tris-HCl, pH6.8 and 0.1% SDS. Both the separating and stacking gels were prepared from a stock solution of 30% acrylamide and 0.8% bisacrylamide and were polymerised by the addition of 0.008% (v/v) tetramethylethylenediamine (TEMED) and 0.08% (m/v) ammonium persulphate. Electrophoresis was carried out using either the 7 x 10 cm or 9 x 10 cm Hoefer Mighty Small™ electrophoresis unit or the larger 16 x 18 cm Hoefer Sturdiel Slab Gel SE 400 unit (Hoefer Scientific Instruments) for improved resolution. The small format gels were run at 120V for 1h30' to 3 hours and the big 16 x 18 cm gels at 70V for 16 to 18 hours. Electrophoresis was carried out in the presence of 1 x TGS buffer (0.025 M Tris-HCl pH8.3, 0.192 M glycine, 0.1% SDS). High range Rainbow Marker™ (AEC Amersham) was resolved on gels as protein size marker.

### **2.2.10. Coomassie Brilliant Blue Staining**

After proteins were sufficiently resolved on the gels they were stained in Coomassie blue stain (0.125% Coomassie blue, 50% methanol, 10% acetic acid) at room temperature for 20 to 40 minutes. The stained gels were then rinsed with tap water to dispose of excess stain and destained in a solution of 5% acetic acid and 5% ethanol.

### **2.2.11. Sucrose Density Gradient Centrifugation**

Sucrose density gradient analysis of baculovirus expressed proteins was utilized to purify the different NS1 constructs based on density and/or size of particles and for analysis of tubule formation. Proteins were expressed on large-scale by infecting monolayers of  $1 \times 10^7$  Sf9 cells in 75cm<sup>3</sup> tissue culture flasks with recombinant baculoviruses as described in section 2.2.8.7. At 72 hours post infection cells were harvested as described in section 2.2.8.8. The collected cells ( $1 \times 10^7$  per flask) were resuspended in 1 ml 1xSTE and the cells mechanically disrupted with a dounce homogenizer. The nuclei were then removed from the cell lysate by low speed centrifugation at 1500 rpm for 5 minutes. The cleared cellular fractions (800 ul) were

loaded onto a 50 to 70% (w/v) discontinuous sucrose gradient. The gradient was set up by layering 800 ul of each percentage sucrose solution in increasing concentrations with 70% at the bottom and 50% at the top in the 5 ml Beckman polyallomer ultracentrifuge tubes i.e. the sucrose solution increased in density with increasing distance down the centrifuge tube. The protein samples were separated on the gradient by centrifugation at 30 000 rpm or 40 000 rpm for 18 hours or 2 hours at 4°C in the Sorvall SW50.1 rotor. After centrifugation the gradients were fractionated into 400 ul aliquots per fraction and the gradient pellets were resuspended in 400 ul 1 x 0.15M STE. The fractions were stored at 4°C.

The protein content of gradient fractions were analysed by either precipitating proteins out of sucrose or by directly loading sucrose on SDS-PAGE gels without protein precipitation. The particulate protein content was precipitated out of sucrose by diluting 200 ul of each fraction in 1200 ul 1 x STE and then precipitating the protein by centrifugation at 5000 rpm for 45 minutes at 4°C. For the analysis of total protein content of each fraction, the fractions were loaded directly on SDS-PAGE gel in the presence of modified 2x protein solvent buffer ( 125 mM Tris-HCl, pH = 6.8., 4% SDS, 10% 2-mercaptoethanol, 0.002% bromophenol blue) without glycerol. The protein content of each fraction was then quantified by the Sigma Gel™ software program (Jandel Scientific) which is used to measure grayscale pixel intensity of protein bands on a SDS-PAGE gel. This information can then be used to calculate relative protein concentrations.

### **2.2.12. Storage Stability Studies**

Proteins for storage stability studies were expressed on large-scale and harvested as described in sections 2.2.8.7 and 2.2.8.8. The collected cells ( $1 \times 10^7$  per flask) from two 75 cm<sup>3</sup> tissue culture flasks were combined and resuspended in 5ml 1x STE. The cell membranes were mechanically disrupted with a dounce homogenizer. The nuclei were then removed from the cell lysate by low speed centrifugation at 1500 rpm for 30 seconds as described in section 2.2.8.8. Following removal of the nuclear fraction the cleared cellular fraction was aliquoted into two 2.25 ml cellular fractions and each fraction layered on top of a solution of 2 ml 30% (w/v) sucrose in a 5 ml Beckman polyallomer ultracentrifuge tube. The sucrose cushion was then centrifuged at 40 000 rpm for 1 hour and 30 minutes at 4°C. Following centrifugation each of the collected protein pellets was resuspended in 2 ml sterile 1 x 0.15M STE and combined to give a total volume of 4 ml. The resuspended proteins were then aliquoted into 500 ul

amounts and the aliquots subjected to different storage conditions. These conditions included overnight storage at  $-70^{\circ}\text{C}$ , flash-freezing the samples in liquid nitrogen and freeze drying. The freeze-dried samples were freeze-dried overnight (12 to 16 hours) in a Freezemobile Twin 6 (Virtis) and re-hydrated by the addition of sterile  $1 \times 0.15 \text{ M}$  STE. The samples stored at  $-70^{\circ}\text{C}$  were defrosted on ice. The treated samples (500  $\mu\text{l}$ ) were then loaded on to 50% to 70% sucrose gradients, centrifuged at 40 000 rpm for 2 hours at  $4^{\circ}\text{C}$ , and fractionated. The proteins from each fraction of the sucrose gradients were analysed by SDS-PAGE and the relative protein concentrations determined using the Sigma Gel™ software program (Jandel Scientific) (section 2.2.11).

### **2.2.13. Microscopy**

#### **2.2.13.1. Transmission electron microscopy (T.E.M)**

A drop (10  $\mu\text{l}$ ) of precipitated, sucrose gradient purified proteins (section 2.2.11) was dispensed onto carbon-coated copper 400-mesh electron microscopy support grid and adsorbed to the surface of the grid for 1 to 5 minutes. Once the specimen had adsorbed to the film surface, the excess sample was blotted from the grid and the grid was placed on a drop of stain solution (2% (w/v) uranyl acetate) for approximately 30 seconds (sample side down). The excess stain was then blotted from the grid and left to dry completely before being observed in a Hitachi H-600 electron microscope at 75 kV.

#### **2.2.13.2. Fluorescence microscopy**

Small-scale protein expression of NS1/eGFP was performed in 6-well plates at cell densities  $1 \times 10^6$  cells per well (section 2.2.8.7). At 72 hours post infection the 6-well plates with adhered infected cells were examined under a Zeiss AxioCam 200 fluorescent microscope. The eGFP was excited at 488 nm and emission detected using a 510/20 bandpass filter. Photographs of the infected cells were taken using a Nikon Digital Camera DXM1200 at magnifications of twenty.

#### **2.2.14. Fluorescence Measurement of NS1/eGFP Protein Samples**

The fluorescence intensities of samples containing NS1/eGFP were measured using a VersaFluor™ Fluoremeter (Bio Rad) with excitation filter at 485- 495 nm and emission filter at 515-525 nm. The protein samples were made up to 1.5 ml in  $1 \times$  STE buffer before taking fluorescence readings.

### 2.2.15. Induction Of Immune Response Against Recombinant NS1 Constructs

Small animal trials were conducted with AHSV6 NS1, NS1/VP2- 40, NS1/VP2 -150 and NS1/eGFP proteins. The proteins were expressed on large-scale in 75cm<sup>3</sup> tissue culture flasks (section 2.2.8.7 and 2.2.8.8) and purified on 50- 70% sucrose gradients (2.2.12). Proteins were then precipitated out of fractions and the fractions containing particulate NS1 (tubules) were pooled. Four groups consisting of three guinea pigs each were immunized subcutaneous with wild-type NS1 or one of the chimeric NS1 constructs. Each animal was immunized with 50 µg of the relevant protein.

All the above groups were immunized with the specified amount and volume of protein in equal amounts of Freund's complete adjuvant. The animals were boosted at day 21 and 42 after the primary immunization

Serum samples were collected at days 14, 21 and 38 after the third boost. The red blood cells were removed from the collected blood samples by low speed centrifugation at 3000 rpm. The sera was the collected and aliquoted into smaller quantities and stored at -20°C.

**Table 2.8.** Immunisation schedule for small animal trials with chimeric NS1 proteins

Group	Construct / sample	Animals/ group	Sample	Sample volume	Adjuvants		
					Day 0 (CFA)	Day 21 (IFA)	Day 42 (IFA)
1	Wild-type	3 guinea pigs	50 µg	1250 µl	1250 µl	1250 µl	1250 µl
2	NS1/VP2-40	3 guinea pigs	50 µg	1250 µl	1250 µl	1250 µl	1250 µl
3	NS1/VP2-150	3 guinea pigs	50 µg	1250 µl	1250 µl	1250 µl	1250 µl
4	NS1/eGFP	3 guinea pigs	50 µg	1250 µl	1250 µl	1250 µl	1250 µl

### 2.2.16. Western Blot Analysis



Baculovirus expressed protein samples were prepared as described in section 2.2.8.7 and 2.2.8.8. Sucrose gradient purified protein samples were prepared as described in section 2.2.11. Protein samples were resolved on 10% or 12% polyacrylamide gels and then transferred onto a nitrocellulose membrane (Hybond C) using the Biorad E.C. 140 Mini Blot Module. Protein transfer was performed at 120 mA for 90 minutes. The membrane was then rinsed in 1 x PBS before commencing blocking in blocking solution [1% (w/v) low fat milk powder in 1x PBS] for 30 minutes at room temperature. Primary antibody solutions were prepared by diluting antiserum in blocking buffer. For all serums a 1 in 30 dilution was used, except for the commercially obtained serum against GFP (Sigma) which was diluted one in a thousand. The blocked membrane was then incubated overnight at room temperature in the presence of the primary antibody solution. The excess primary antibodies were then removed by three washes in wash buffer (0.05% Tween 20 in 1 x PBS) before incubation at room temperature for 1 hour in presence of the secondary antibody labeled with horseradish peroxidase. The secondary antibody, peroxidase-conjugated protein A (ICN) was diluted to a 1 in 250 dilution in blocking solution. Following this, the membrane was washed in three changes of washing buffer and once in 1 x PBS to remove excess secondary antibody. The enzyme substrate (prepared by dissolving 60 mg 4-chloror-1-naphtol in 20 ml ice cold methanol and adding to a solution of 60 ul of hydrogen peroxide in 100 ml 1 x PBS) was then added to the membrane and incubated at room temperature until the protein bands became visible. The detection reaction was stopped by rinsing the membrane in dH<sub>2</sub>O and the membranes were then air-dried.

## 2.3 RESULTS

This study aims to investigate the effect on particle formation of AHSV6 NS1 modified by the internal insertion of differently sized immunogenic regions from AHSV9 VP2 and the whole eGFP gene by:

- Identifying an appropriate site for peptide insertion in the NS1 amino acid sequence.
- Cloning of DNA sequence, coding for immunogenic regions of VP2 and marker protein eGFP, into NS1 gene.
- Expression of recombinant genes in the baculovirus expression system.
- Stability and structure analysis of expressed recombinant proteins by sucrose gradient analysis and microscopy.

The second aim of this study is the investigation of the potential of NS1 tubular structures to present an immunogenic region of AHSV VP2 and the marker protein, eGFP, to the immune system in a manner that elicits an epitope specific immune response. This was achieved by performing:

- Small mammal trials with baculovirus expressed chimeric NS1 proteins.
- Western blot analysis using the antiserum obtained from small mammal trials as well as western blot analysis using serums generated against the VP2 inserts presented in another presentation system and the commercially bought antibodies specific for the eGFP protein.

The eGFP protein insert will not only serve as a reporter protein for monitoring gene expression but fluorescence of the recombinant NS1-eGFP protein will also give an indication of tubule stability after inserting 240 amino acids, and very importantly the stability of peptides inserted into the C-terminus region of the NS1 protein. The use of a GFP-type protein as insert has the additional benefit that antibodies against this protein is commercially available and this becomes increasingly important when evaluating the immunological presentation of a peptide on the surface of particulate protein vector.

### **2.3.1. Sequence Verification Of AHSV6 NS1 Gene**

The AHSV NS1 gene cloned into the pBS vector (Maree, 2000) was sequenced by automated DNA sequencing and aligned (section 2.2.2) to the published AHSV6 NS1 sequence (Maree, Huisman, 1997) as well as the published sequences for NS1 of AHSV4 and AHSV9 (appendix A-1). Sequencing revealed 10 nucleotide differences between the published AHSV6 NS1 and sequenced gene sequence (appendix A-1). Due to an insertion at nucleotide position 998 (indicated in green in appendix A-1) a frame shift mutation occurs, which is corrected by a deletion at nucleotide position 1074 (indicated in pink in appendix A-1). This specific insertion and deletion also occurs in the NS1 gene of the AHSV4 NS1 gene. The C-terminal region of the gene which was the target for inserting foreign peptides is unaffected by these deletions.

### **2.3.2. Identification Of a Site On AHSV6 NS1 Protein For Peptide Insertion based on physico-chemical properties**

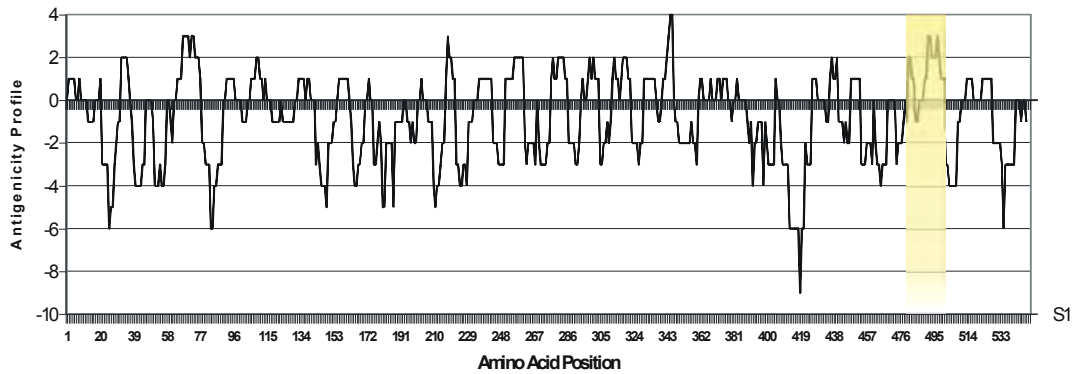
The ANTHEPROT software package (Geourjon, Deleage, 1995; Geourjon *et al.*, 1991) was used to compile a hydrophilicity (Hopp, Woods, 1981; Hopp, Woods,

1983) and antigenicity (Welling *et al.*, 1985) profile of AHSV6 NS1. As shown in the hydrophilicity plot of NS1 (Figure 2.2) NS1 has a net hydrophobicity and is characterised by alternate regions of hydrophobicity and hydrophilicity. This characteristic distribution of hydrophilic and hydrophobic regions can be explained in context of the tubule structure formed by NS1 proteins. In these tubules the hydrophobic regions of the proteins are located within the tubule and the hydrophilic regions exposed on the surface of the protein.

A region of antigenicity was identified within one of the hydrophilic regions in the C-terminal region of the protein based on a comparison of the hydrophilicity and antigenicity profiles of the AHSV6 NS1 protein (Figure 2.2). Peptides inserted into this region should in theory be displayed on the surface of the tubules formed by NS1 proteins. This region corresponds to amino acid position 470-504 and it is encoded by a sequence that includes a unique *Xho*I site at position 1414-1420.

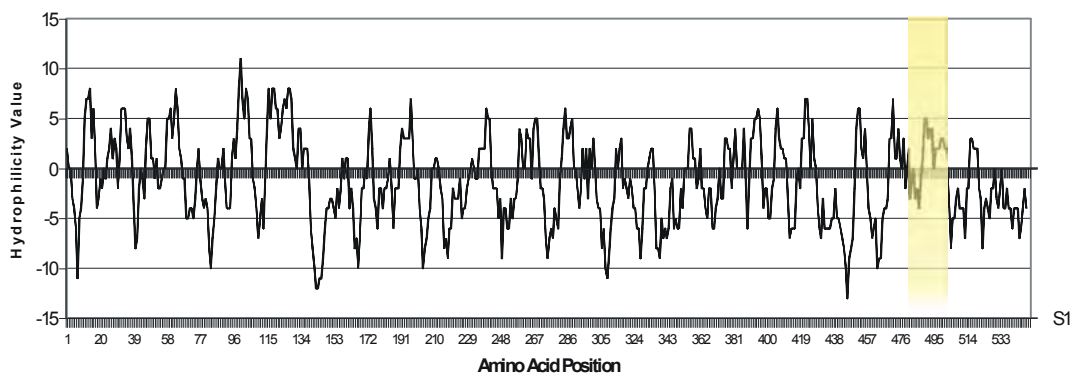
**A**

**Antigenicity Profile of AHSV6 NS1**



**B**

**Hydrophilicity Profile of AHSV6 NS1**

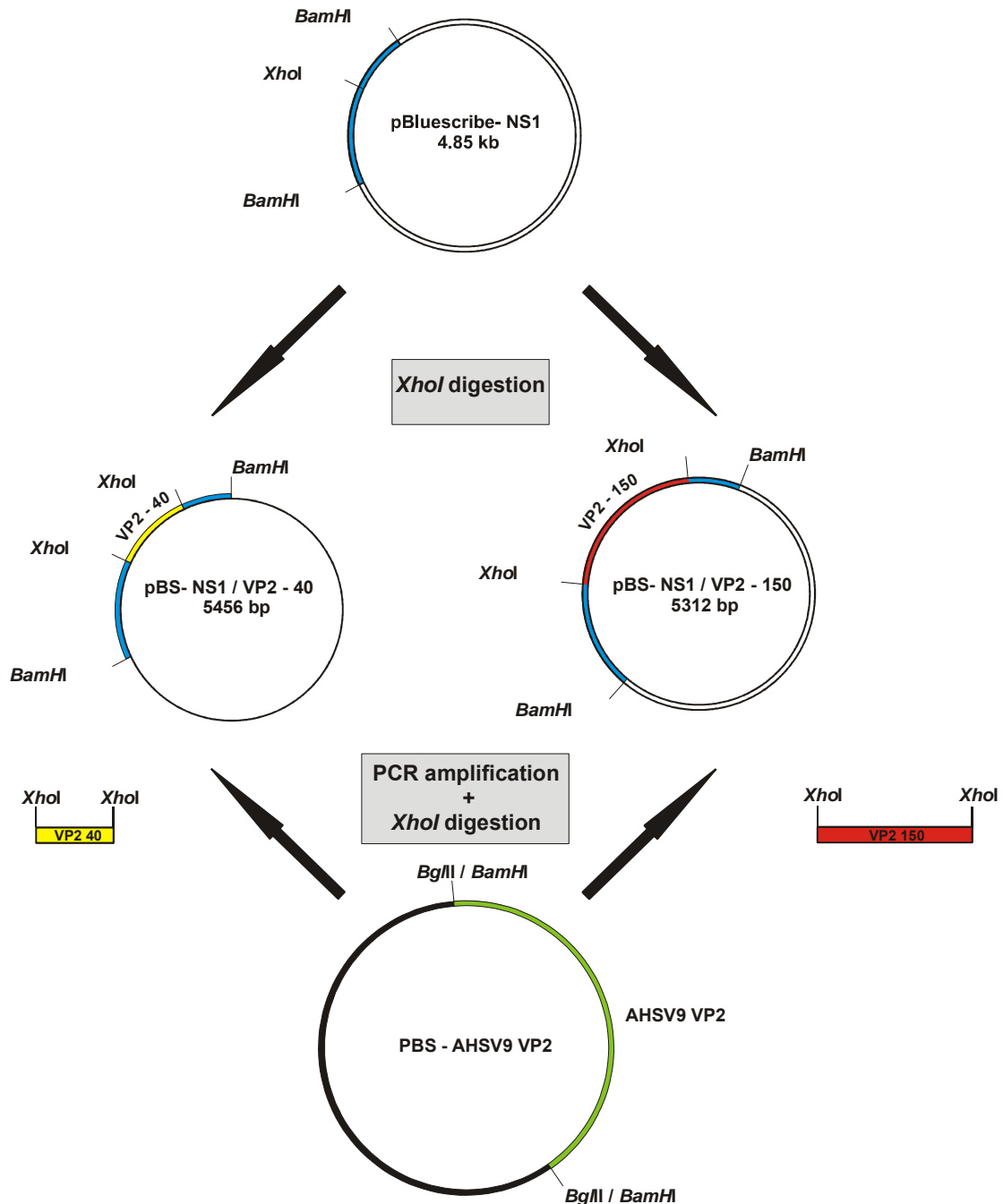


**Figure 2.2.** Comparison of the Antigenicity (A) (Wellington et al., 1988) and the hydrophilicity profile (B) (Hopp and Woods, 1981; Hopp and Woods, 1983) of AHSV6 NS1. The highlighted areas indicate overlapping regions of antigenicity and hydrophilicity in the C-terminal region of the protein. This region is targeted for peptide insertion.

### 2.3.3. Construction Of Chimeric NS1 genes

The pBluescribe plasmid vector, pBS-NS1 carrying the coding region of the AHSV6 NS1 (Maree, 2000) was used for the construction of the chimeric NS1 genes. The

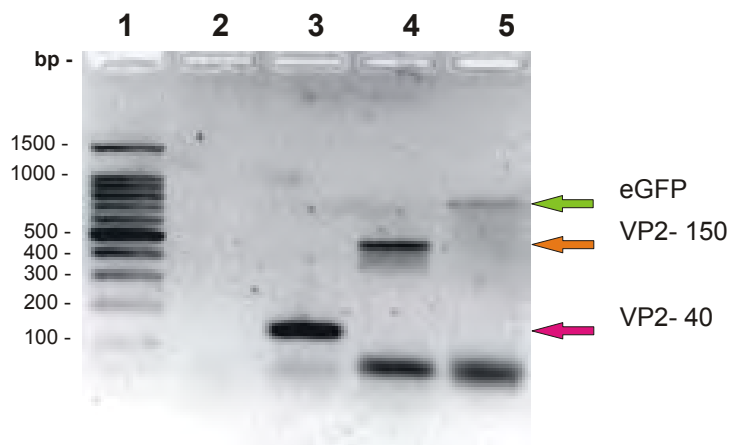
basic cloning strategy for the construction of the two recombinant NS1 genes containing AHSV9 VP2 insert is summarized in Figure 2.3. The same strategy was used for the construction of recombinant NS1/eGFP gene.



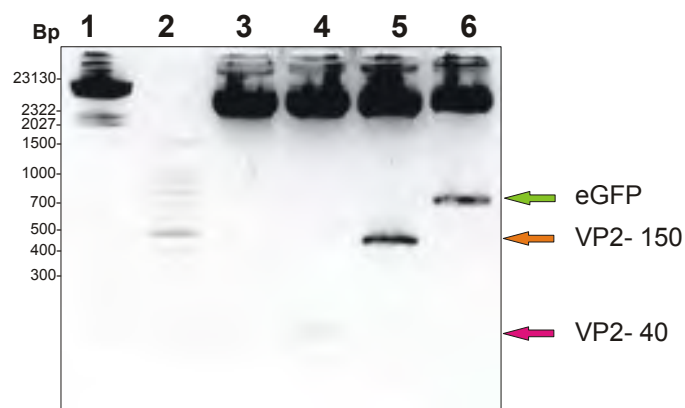
**Figure 2.3.** Cloning strategy for the construction of pBluescribe- NS1/VP2- 40 and pBluescribe- NS1/VP2- 150.

The cDNA clone of AHSV9 segment 2 in plasmid pBS9.2Hyb (Napier, 1999) was used as template for the PCR amplification of the 120 and 450 base pair regions (Table 2.2). The primers were designed so that *XhoI* sites were incorporated at the

ends of the amplified regions. The eGFP gene in the PGEM t-easy™ vector, obtained from L. Burger of the department of Microbiology, University of Pretoria, was used as template for the amplification of the complete eGFP gene, including start codon but excluding the stop codon. The primers are summarized in Table 2.4. As can be seen in Figure 2.4 the amplified DNA fragments corresponded to the expected sizes of 135 bp (40 aa VP2 region), 468 bp (150 aa VP2 insert) and 732 bp (eGFP gene insert).



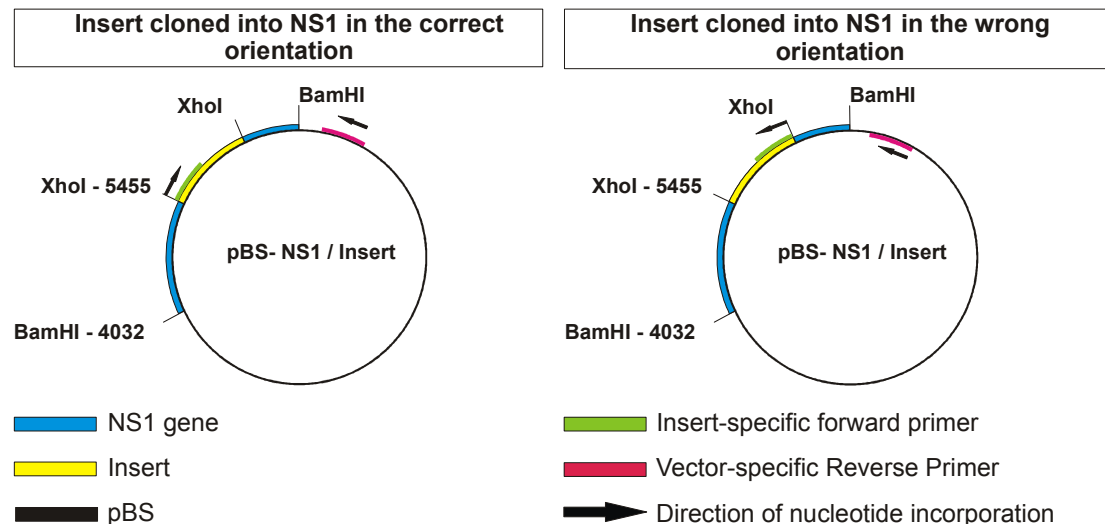
**Figure 2.4.** Agarose gel electrophoresis of the PCR amplification products of AHSV9 VP2-40 insert (lane 3), AHSV9 VP2-150 insert (lane 4) and the eGFP gene (lane 5). The samples were run along with a 100 bp DNA ladder size marker (lane 1) and a negative control of a PCR reaction lacking DNA template (lane 2). Samples were loaded on a 2% agarose gel.



**Figure 2.5.** Agarose gel electrophoresis of *Xho*I digestions of pBS- NS1 (lane 3), pBS-NS1/VP2-40 (lane 4), pBS-NS1/VP2-150 (lane 5) and pBS-NS1/eGFP (lane 6). The samples were run along with a 100 bp DNA ladder size marker (lane 2) and molecular weight marker II (lane 1). Samples were loaded on a 2% agarose gel.

The vector (pBS-NS1) and inserts were prepared for cloning by digestion with *XhoI*. Cloning was carried out as discussed in section 2.2.5. Recombinant plasmid clones were selected by *XhoI* digestion of the pBS-NS1 vector and putative recombinant clones. Restriction enzyme digestion produced an expected linear 4.85 kbp band and the recombinant vectors also produced bands of 129 bp, 462 bp and 726 bp, respectively (Figure 2.5).

PCR was used to determine the orientation of inserts using the insert-specific forward primer and vector-specific reverse primer that binds in the MCS downstream of the gene (Table 2.6). When the insert is cloned in the correct orientation the PCR product will correspond to the size of the insert, C-terminal 240 nucleotides of the NS1 gene and a 98 nucleotide region in the MCS of the vector. A recombinant gene that contains the insert in the incorrect orientation is not expected to give an amplification product. The PCR strategy is summarized in Figure 2.6. As predicted, VP2-40, VP2-150 and eGFP clones with the insert in the correct orientation produced PCR products of 473 bp, 806 bp and 1070 bp, respectively. The template DNA can be observed as single bands at the top of lanes 3, 4 and 5 (Figure 2.7). The recombinant constructs were designated pBS- NS1/ VP2- 40, pBS- NS1/ VP2- 150 and pBS- NS/ eGFP, respectively.



**Figure 2.6.** Theoretical representation of primer binding to insert in both the correct and wrong orientation within the NS1 gene. The direction of nucleotide incorporation by the polymerase enzyme during a PCR reaction is indicated by the black arrows.



**Figure 2.7.** Agarose gel electrophoresis of PCR orientation determination of inserts in recombinant pBS- NS1/VP2-40 (lane 3), pBS- NS1/VP2-150 (lane 4) and pBS- NS1/ eGFP (lane 5) constructs. Lane 1 represents a 100 bp DNA ladder size marker and lane 2 contains the negative control of a PCR reaction without DNA template.

#### 2.3.4. Nucleotide Sequence Determination

External plasmid-specific and internal NS1-specific primers (Table 2.3) were used for automated sequencing to verify the sequence of the chimeric NS1. The sequence alignments confirmed the presence of insert in the *XhoI* site in the correct orientation and reading frame (Appendices A-2 and A-3). The corresponding amino acid sequences were then aligned to the AHSV6 NS1 the amino acid sequences using the ABI PRISM Navigator™ program to verify the integrity of chimeric NS1 proteins (Appendices A-4 and A-5).

#### 2.3.5. Physico-chemical properties of the Chimeric NS1 proteins

The effect of the inserts on the hydrophilicity profile of NS1 was modeled using the Hopp and Woods predictive method (Hopp, Woods, 1981; Hopp, Woods, 1983). The hydrophilicity profiles for NS1/VP2-40, -150 and NS1/eGFP are shown in Figures 2.8 to 2.10, respectively. As expected for two identified immunogenic regions, the VP2-40 and VP2-150 inserts are hydrophilic as can be seen from their positive hydrophilic values (Table 2.8). The insertion of the VP2 inserts into the NS1 protein causes the formation of distinctive hydrophilic region within the C-terminal of the NS1 protein (Figures 2.8 and 2.9) and increases the average hydrophilicity of the recombinant protein (Table 2.8). It is important to note that overall the proteins are still hydrophobic as can be seen from the negative average hydrophilicity value (Table 2.8). With the NS1 protein being largely hydrophobic, the insertion of these relatively large hydrophilic regions may effect the folding of the protein into its native form. The effect of these hydrophilic inserts might be minimal due to the hydrophilic nature of

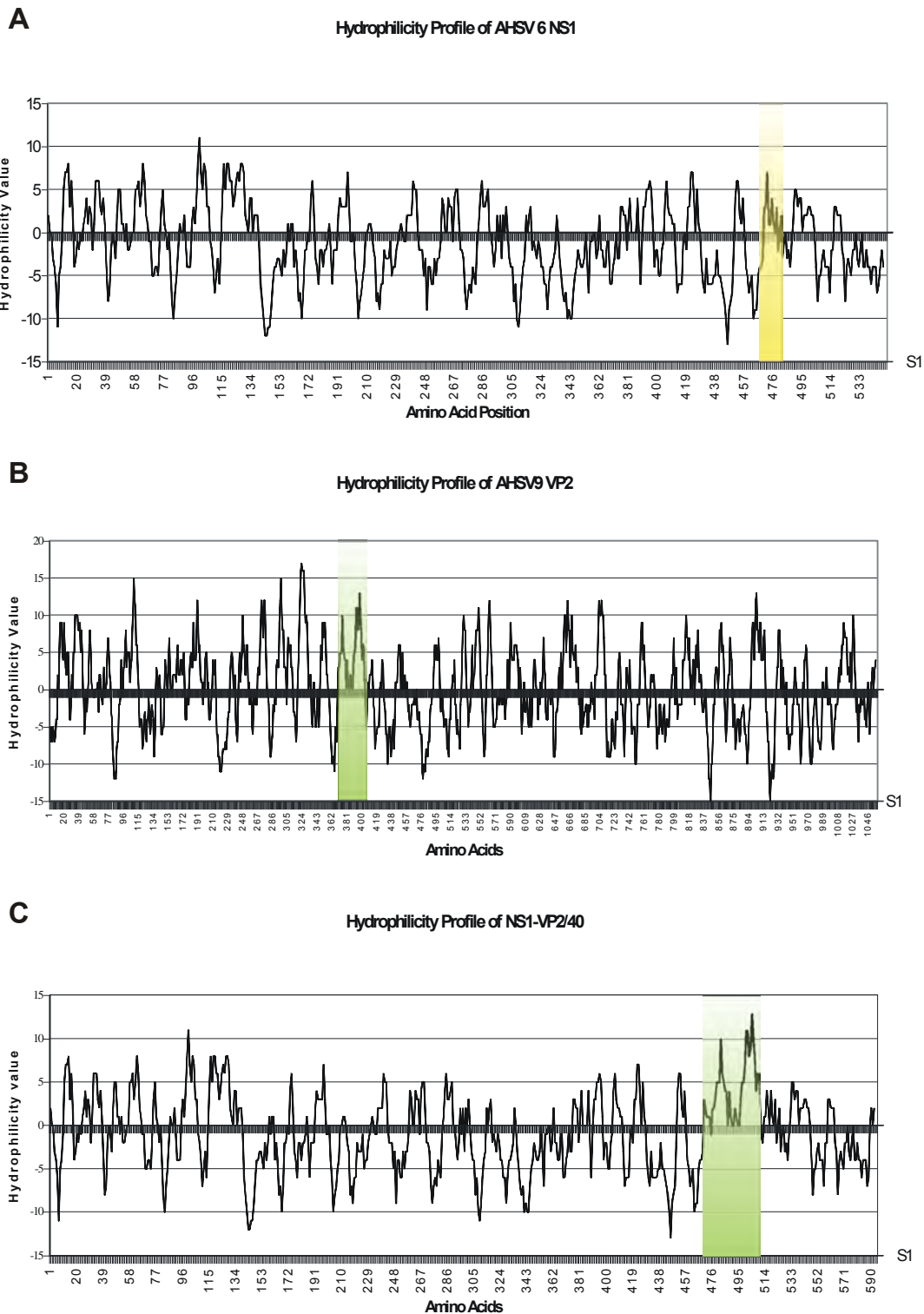


the insertion site on NS1 and it is thought that the hydrophilic insert will be displayed on the surface of the NS1 protein (section 2.3.2) without interfering much with the NS1 protein structure. It is possible that due to the increased size of the hydrophilic region in the chimeric NS1 proteins, the protein folding could be less dense due to steric hindrance causing an overall increase in the diameter of assembled tubules.

In contrast to the VP2-inserts, the eGFP gene insert is hydrophobic (negative hydrophilicity value) (Table 2.8), but the protein, as the NS1 protein, is characterised by hydrophilic regions that are intermittently dispersed over the length of the protein (Figure 2.10). These hydrophilic regions are exposed on the surface of the barrel-like GFP structure (Ormo *et al.*, 1996; Wachter *et al.*, 1997; Yang *et al.*, 1996). The eGFP forms a distinct region in the NS1/eGFP protein (Figure 2.10) the average hydrophobicity of the NS1/eGFP protein is slightly higher when compared to that of NS1 due to the hydrophobic eGFP insert (Table 2.8).

**Table 2.8.** Predicted hydrophilicity values of the VP2 and eGFP inserts and the NS1 Protein Constructs (Hopp, Woods, 1981; Hopp, Woods, 1983)

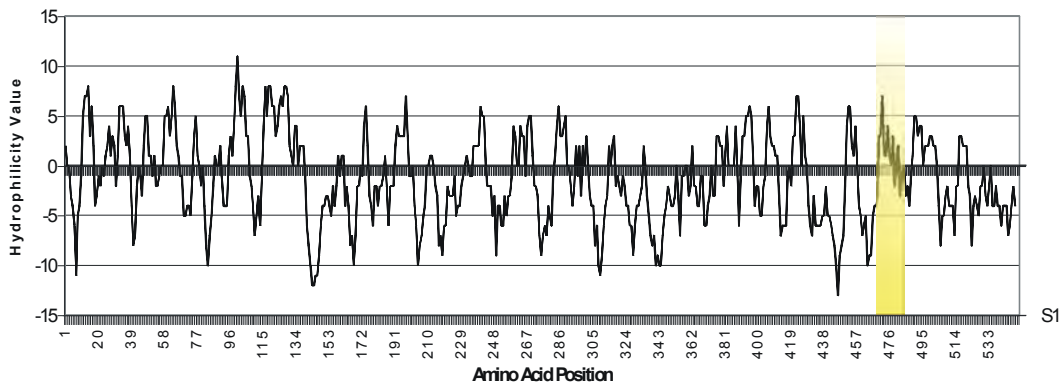
Protein / Insert	Predicted Hydrophilicity Value	
	Total	Average
AHSV6 NS1	-665	-1.21
VP2- 40 insert	+173	+4.02
VP2-150 insert	+359	+2.33
eGFP protein	-72	-0.30
NS1/VP2- 40	-500	-0.85
NS1/VP2- 150	-291	-0.41
NS1/eGFP	-730	-0.92



**Figure 2.8.** Hydrophilicity profiles of AHSV6 NS1 (A), AHSV9 VP2 (B), and NS1/VP2-40 (C) (Hopp and Woods, 1981; Hopp and Woods, 1983). The region highlighted in yellow indicates the peptide insertion site in the AHSV6 NS1 protein. Highlighted green regions indicate the area of AHSV9 VP2 inserted into AHSV6 NS1.

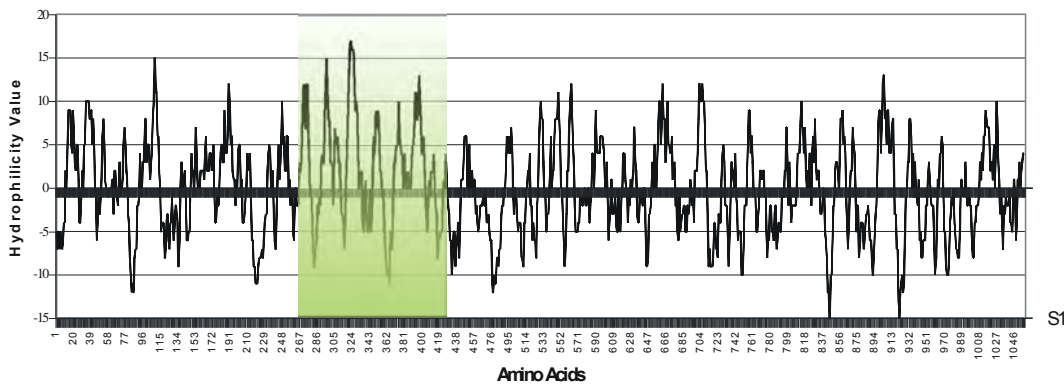
**A**

**Hydrophilicity Profile of AHSV6 NS1**



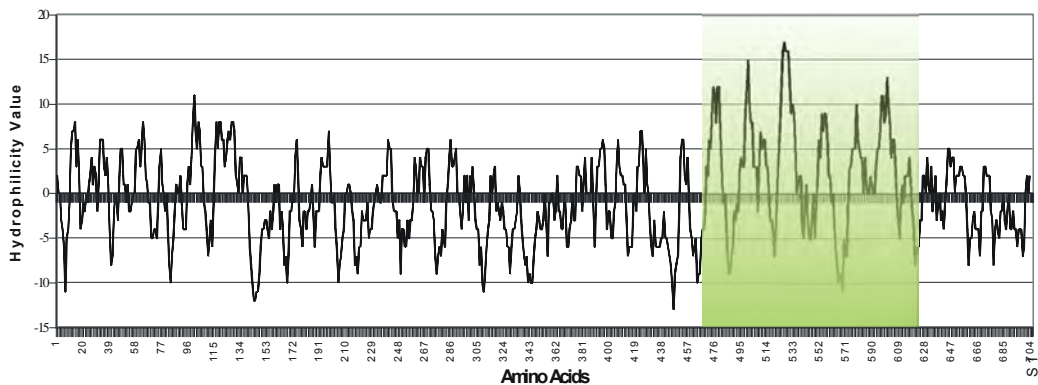
**B**

**Hydrophilicity Profile of AHSV9 VP2**

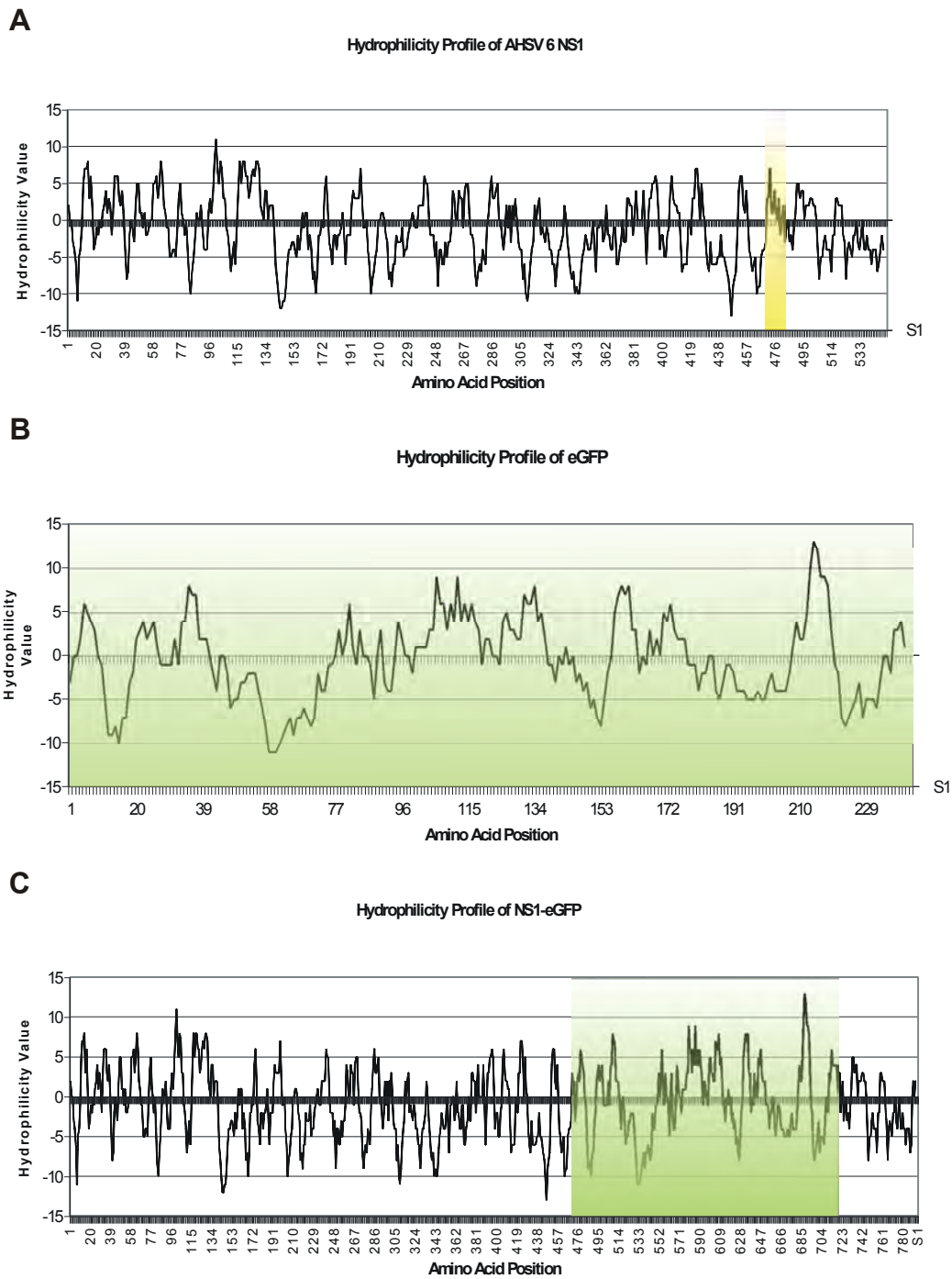


**C**

**Hydrophilicity Profile of NS1-VP2/150**



**Figure 2.9.** Hydrophilicity profiles of AHSV6 NS1 (A), AHSV9 VP2 (B), and NS1/ VP2- 150 (C) (Hopp and Woods, 1981; Hopp and Woods, 1983). The region highlighted in yellow indicates the peptide insertion site in the AHSV6 NS1 protein. The highlighted green regions indicate the area of AHSV9 VP2 that is inserted into AHSV6 NS1.



**Figure 2.10.** Hydrophilicity profiles of AHSV6 NS1 (A), eGFP (B), and NS1/eGFP (Hopp and Woods, 1981; Hopp and Woods, 1983). The region highlighted in yellow indicates the peptide insertion site in the AHSV6 NS1 protein. The highlighted green regions indicate the area of eGFP that is inserted into AHSV6 NS1.

### **2.3.6. Baculovirus Expression Of NS1 Constructs**

#### **2.3.6.1. Construction of recombinant baculovirus transfer plasmids**

In order to express the chimeric NS1 genes using the Bac-To-Bac system it was necessary to clone the chimeric genes into the baculovirus transfer plasmid pFastBac1. The cloning strategy for pFB-NS1 constructs is summarized in Figure 2.11. The pFB vector was linearised with *Bam*HI to prepare it for insertion of the recombinant NS1 genes. The recombinant NS1 genes were cut out of the pBS plasmids by digestion of pBS- NS1/VP2- 40, pBS-NS1/VP2-150 and pBS-NS1/eGFP plasmids with *Bam*HI. Restriction enzyme digestion of pBS plasmids produced fragments corresponding to the size of the plasmid (3.2 kbp) and the excised NS1/VP2-40 (1790 bp) or NS1/VP2-150 (2123 bp) or NS1/eGFP (2387 bp) gene (Figure 2.12). The DNA fragments were then excised from the agarose gel, cloned into the prepared pFB vector, and the recombinant colonies were screened by *Bam*HI digestions to confirm the presence of the recombinant NS1 genes (results not shown). *Hind*III digestions were carried out on the *Bam*HI-selected samples to confirm that genes were cloned in the correct orientation into pFB relative to polyhedrin promoter. Selected samples were sequenced to confirm insert orientation and sequence integrity.

#### **2.3.6.2. Construction and selection of recombinant baculoviruses**

Recombinant baculoviruses were constructed as described in section 2.2.8. Following transfection, putative recombinant baculoviruses were used for small-scale infections (section 2.2.8.5) and total, unpurified cell lysates from these infections were then analysed by SDS-PAGE (Figure 2.13). The protein constructs were designated WT-NS1, NS1/VP2-40, NS1/VP2-150 and NS1/eGFP and have predicted molecular weights of 63 kDa, 69 kDa, 81 kDa and 91 kDa, respectively. The SDS-Page gel estimated MW of the NS1 fusion proteins are higher than that of the predicted value. Discrepancies in predicted molecular weight from its deduced amino acid sequence and apparent molecular weights have also been reported for BTV NS1 proteins (Monastyrskaya *et al.*, 1995).

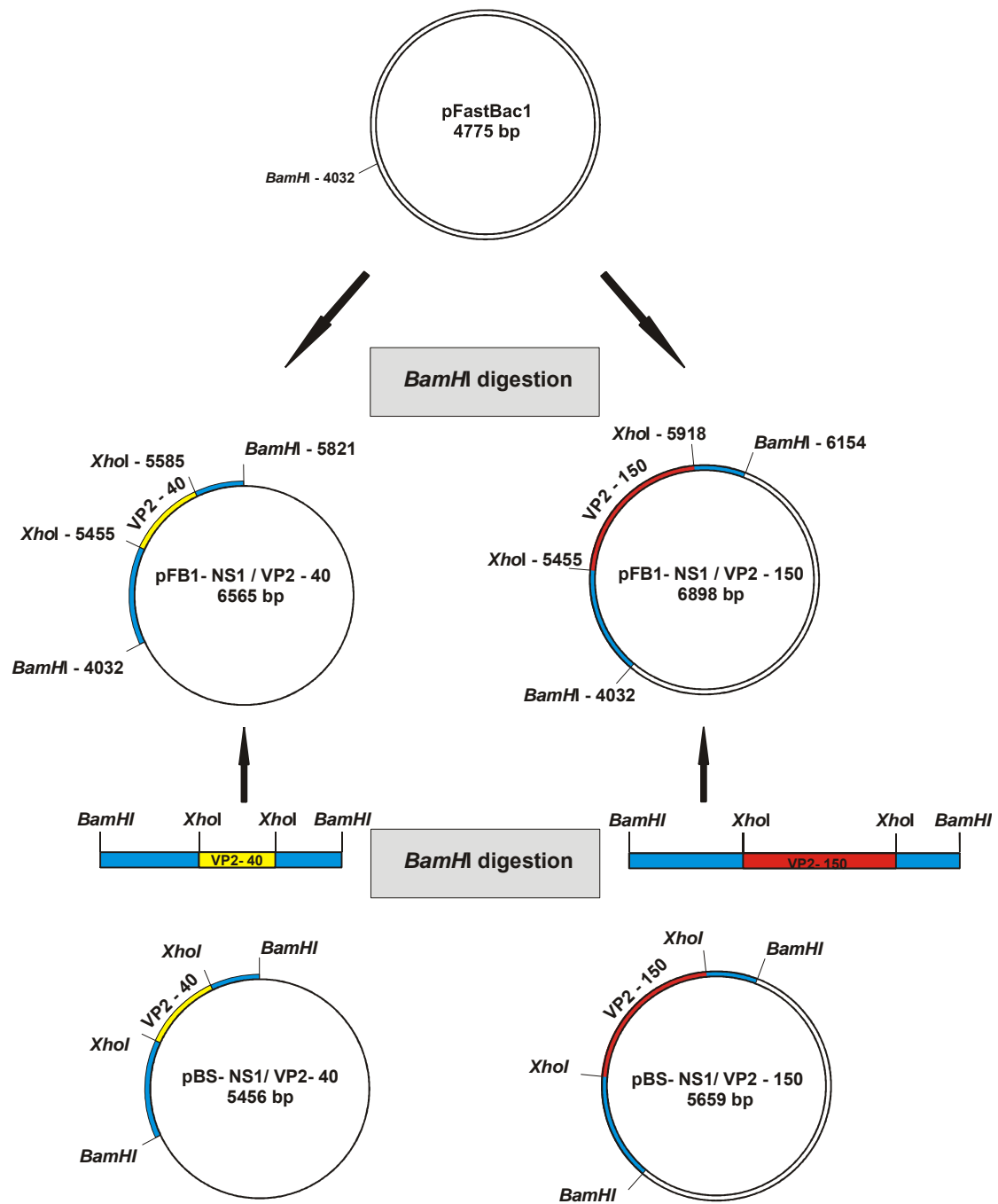
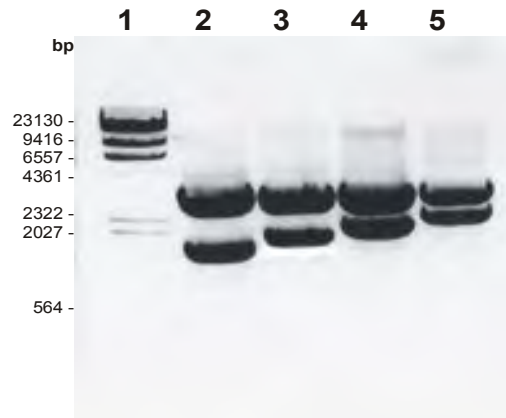
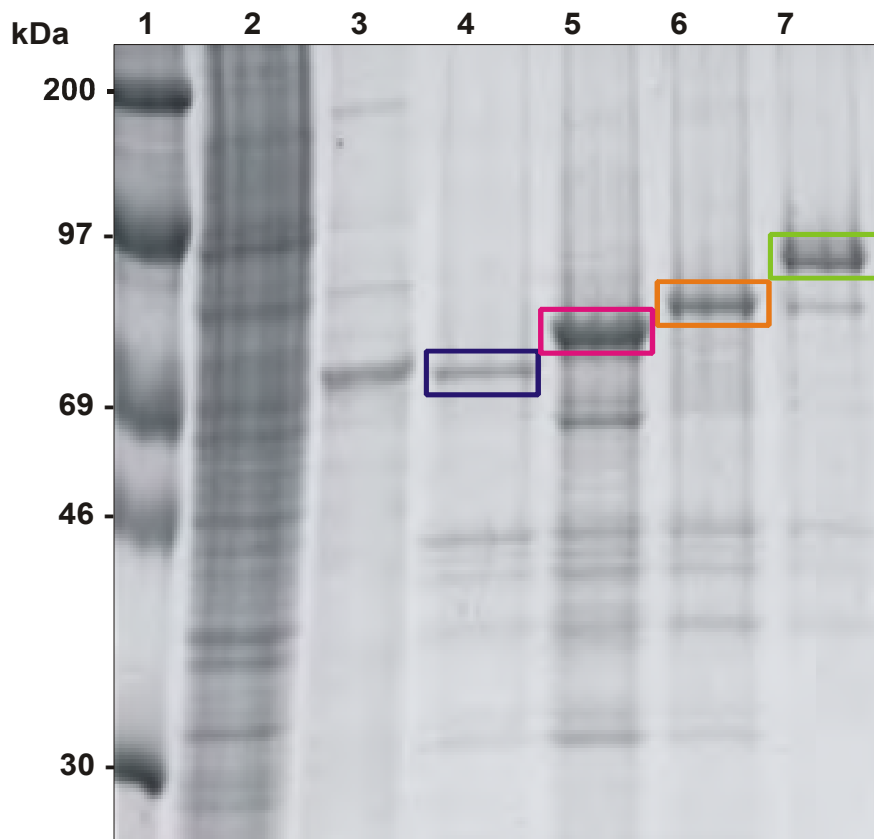


Figure 2.11. Cloning strategy for the construction of pFastBac1- NS1/VP2- 40 and pFastBac1- NS1/VP2- 150.



**Figure 2.12.** Agarose gel electrophoresis of BamHI digestions of pBS-NS1 constructs. BamHI digestions of pBS-NS1 (lane 2), pBS- NS1/VP2-40 (lane 3), pBS- NS1/VP2- 150 (lane 4) and pBS- NS1/ eGFP (lane 5 ) as well as the size marker MWII (lane 1) was loaded on a 1% agarose gel.



**Figure 2.13.** SDS-PAGE analysis of the expression of AHSV6 NS1 and chimeric NS1 constructs. Lane 1 represents the protein size marker and lanes 2 and 3 show uninfected and wild-type baculovirus infected Sf9 cell extracts, respectively. Lanes 4, 5, 6 and 7 contain cell extracts from Sf9 cells infected with AHSV6 NS1 (blue box), NS1/VP2-40 (pink box), NS1/VP2-150 (orange box) and NS1/eGFP (green box) recombinant baculovirus, respectively. The colored boxes indicate the position of the recombinant proteins.

### 2.3.7. Density Gradient Centrifugation

Fusion proteins were analyzed by sucrose gradient centrifugation to investigate the effect of inserting different peptides on the structure of AHSV6 NS1. Preliminary results indicated that the NS1 protein complex has a low particle density and that the fractionation of the chimeric tubular particles on 70-50% sucrose gradient was determined by a combination of both density and size of the NS1 tubular particles.

To confirm these preliminary results, native AHSV6 NS1 was analyzed on two identical discontinuous 50 – 70% (w/v) gradients centrifuged at 40 000 rpm for either 2 hours or 18 hours. Proteins were prepared for sucrose density analysis as described in section 2.2.11. The fractions were collected from the bottom of the gradients in 400 ul aliquots, resulting in 12 to 13 fractions (including pellet fraction). A sample of each sucrose gradient fraction was analysed by SDS-PAGE. The intensity of the NS1 protein band of each fraction on the SDS-PAGE gel was quantified by the SigmaGel™ software package (Jandel Scientific). The distribution of the proteins on the sucrose gradient as resolved on SDS-PAGE gel was then converted to a graphic form to visually present the distribution of the protein across all the fractions of the sucrose gradients. The proteins are quantified as a percentage of the total specified protein content across all the fractions in a sucrose gradient. Each experiment was repeated at least three times and average values were calculated.

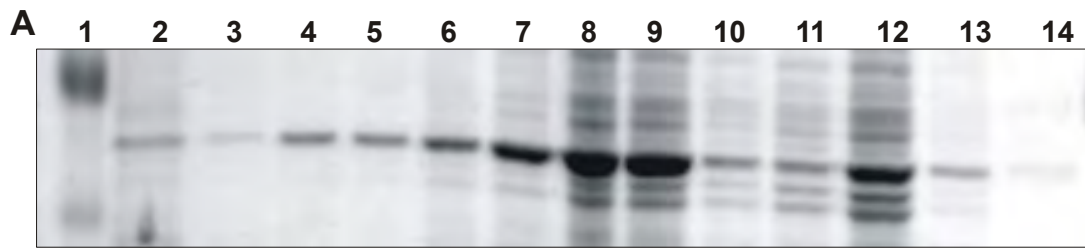
Figure 2.14 shows the distribution of wild type NS1 proteins in a sucrose gradient after centrifugation at 40 000 rpm for 2 hours and 18 hours, respectively. It is evident there is a heterogeneous distribution of NS1 particles on the gradients after 2 hours (A) centrifugation and 18 hours (B) centrifugation. After 2 hours centrifugation (Figure 2.14 A) a proportion of the protein is distributed across all the fractions of the gradient with the greater proportions occurring in two peaks, one peak at the top of the gradient (fraction 11) and the other bigger peak at a higher sucrose density position lower in the gradient. Prolonged centrifugation of the NS1 protein for 18 hours causes a more homogenous distribution of protein with the majority of the proteins distributed across the first 9 fractions (Figure 2.14 B). These results show that the larger sized proteins and protein complexes such as NS1 tubules have a higher sedimentation velocity, hence achieving density equilibrium faster than smaller sized proteins such as NS1 dimers due to the action of centrifugal force. These larger proteins do not necessarily have a greater density than smaller proteins as is the case for NS1 that has a greater density than NS1 tubules due to the internal lumens



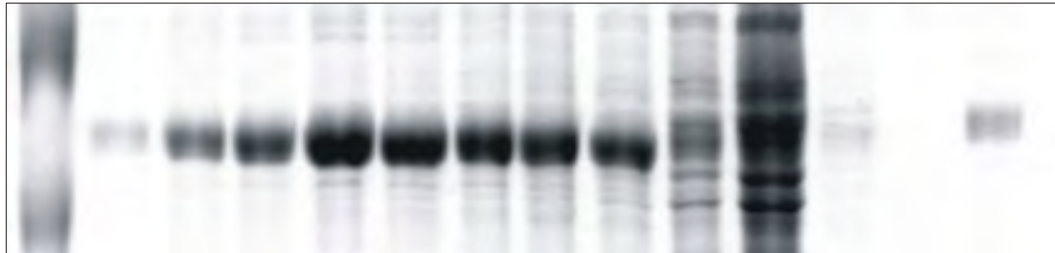
that form in these structures. In Figure 2.14 (A) the NS1 proteins peak in the top fractions of the gradient such as fraction 12 are presumed to contain protein with a smaller physical size and not necessarily lower density that take longer to migrate through the sucrose gradient due to their smaller sedimentation velocity. The distribution profile obtained after a short period of centrifugation is determined both by the size of the particle and the density as seen in Figure 2.14 (A). As can be seen in Figure 2.14 (B), after a longer period of centrifugation a more heterogeneous distribution is observed. This downward shift of protein particles following longer centrifugation can also partly be attributed to the changes in density that occur in the sucrose gradient due to centrifugation. The proportion NS1 proteins that remain in the top fractions of the gradient after prolonged centrifugation can possibly be attributed to the association of a proportion of the NS1 proteins with membrane complexes as the protein lysates used for centrifugation were not detergent treated. These NS1-membrane-complexes will have a lower overall density that causes them to remain in the top lower density fractions of the gradient.

It seems that even after 18 hours centrifugation there still are small high density particles that move through the gradient according to rate zonal centrifugation. The larger NS1 tubules/aggregates that have a lower density move quickly and reach their equilibrium density quickly and then stop.

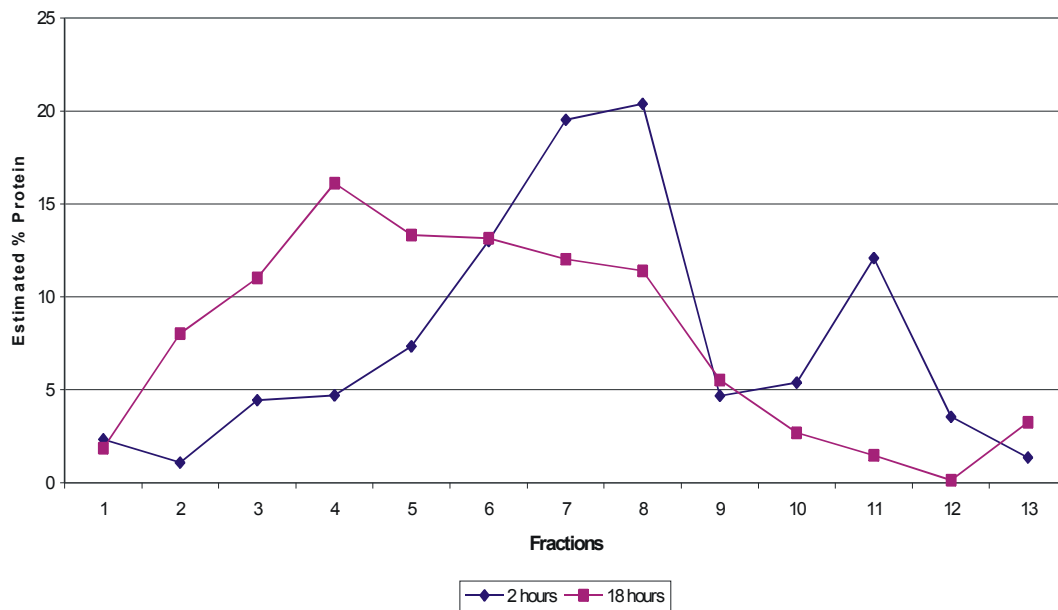
### 2 Hours Centrifugation



### 18 Hours Centrifugation



**Comparative Particle Distribution of NS1 in Sucrose Gradient following 2 hours and 18 hours Centrifugation**

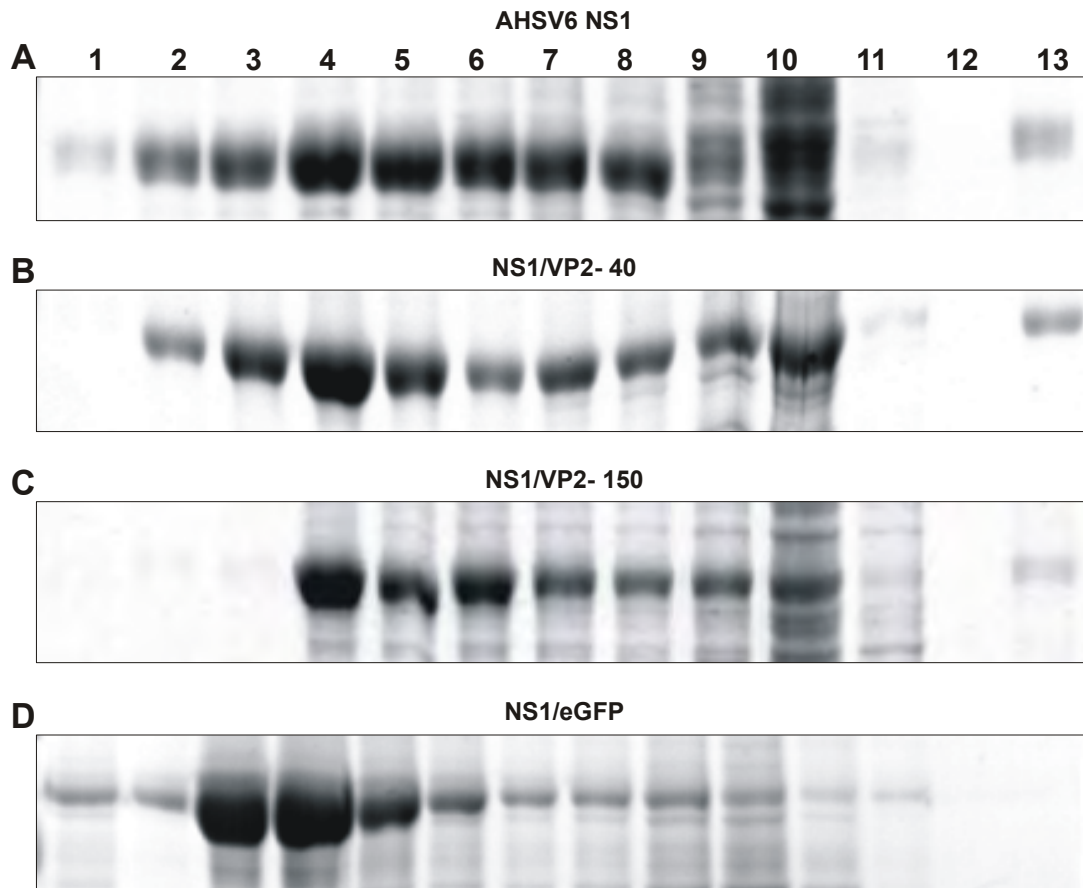


**Figure 2.14.** Comparative distribution of baculovirus expressed AHSV6 NS1 in a 50% to 70 % sucrose gradient under centrifugation conditions of 40 000 rpm for 2 hours and 40 000 rpm for 18 hours. Figures (A) and (B) show the SDS-PAGE analysis of NS1 gradients centrifuged for 2 hours or 18 hours, respectively. Lanes 1 represent a protein size marker, lanes 2 represent the pellet of the gradient and lanes 3 to 14 represent the gradient fractions from highest to lowest density. The graph shows the estimated percentage protein (of total NS1) in each fraction.

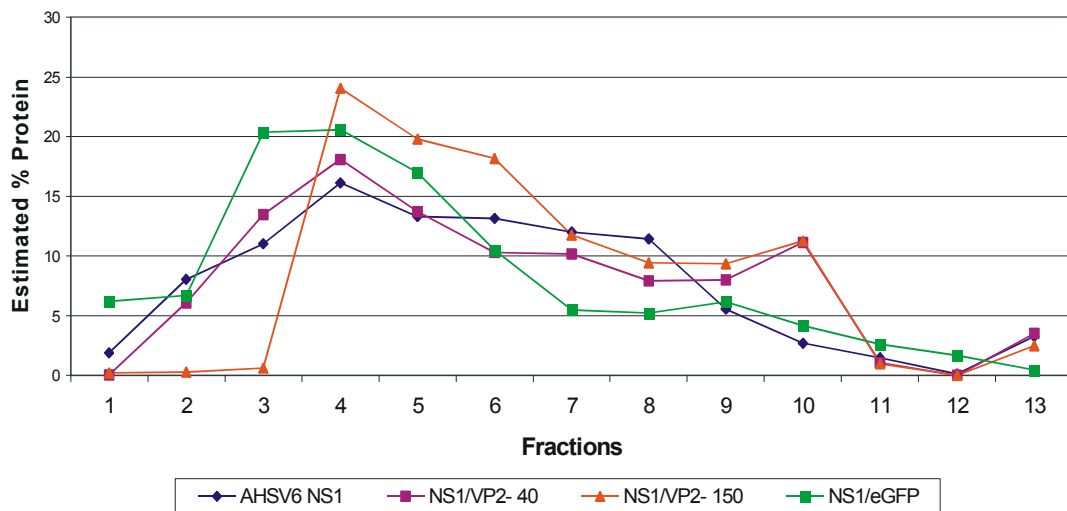
Comparative sucrose density gradient studies were performed for all the chimeric NS1 constructs and native AHSV6 NS1 to study the particulate structures formed by the chimeric NS1 proteins and the effect of insertions on protein structure. Sucrose gradient analysis was performed as described above. All sucrose gradients were centrifuged at 40000 rpm for 18 hours. As can be seen from Figure 2.15 all the protein constructs, native and chimeric, show a relatively heterogeneous protein distribution when separated on sucrose gradient. For all constructs the majority of proteins occur in the bottom two thirds of the gradients that correspond to the higher density region of the gradient, which suggests that the chimeric proteins are present in a particulate form (Huismans, Els, 1979; Maree, Huismans, 1997; Monastyrskaya *et al.*, 1995; Owens *et al.*, 2004; Urakawa, Roy, 1988). The heterogeneity of the S values on sucrose gradient is due to the levels of structural assembly of tubular structures (as discussed in section 1.2.2.2) that differ in density.

The chimeric proteins, NS1/VP2-40 and -150, show a decrease in protein concentration in fractions 1 and fractions 1 to 3, respectively, when compared the sucrose gradient distribution of native AHSV6 NS1. These two proteins do not form the higher density protein particles observed for native NS1. These differences in density sedimentation of the proteins can probably be attributed to the hydrophilic nature of the VP2 inserts that could cause a decrease in the density of tubule assembly (as discussed in section 2.3.5), hence less dense particles that reach density equilibrium at a lower density than native NS1 tubules. The characteristic tubule lumen also affects the density of NS1 tubules and if the insert affects the packaging of NS1 subunits into tubules, as discussed above, the size or volume of the lumen could change, in turn influencing the density of tubules. The insertion of the hydrophilic particles could also decrease the aggregation of the chimeric NS1 proteins into the large high density amorphous aggregates that are observed for native NS1 (Monastyrskaya *et al.*, 1995).

The NS1/eGFP and the wild type NS1 protein have very similar sucrose gradient profiles. As can be seen from Figure 2.15, the greatest proportion of NS1/eGFP protein occur in the bottom four fractions of the gradient and sediment at a position of slightly higher density than the wild type NS1 protein. In terms of the hydrophobic nature of the insert this is unexpected, but the higher density of the protein can be explained in terms of the tight barrel-like nature of the eGFP insert that could cause an overall increase in the density of the NS1/eGFP protein (Ormo *et al.*, 1996; Wachter *et al.*, 1997; Yang *et al.*, 1996).



### Comparative Particle Distribution of NS1 Constructs on Sucrose Density Gradient



**Figure 2.15.** Particle distribution of AHSV6 NS1 (A), NS1/VP2-40 (B), NS1/VP2-150 (C) and NS1/eGFP in a 50- 70% sucrose gradient shown by SDS-PAGE analysis and graphic representation. Proteins were separated under centrifugation conditions of 40 000 rpm for 18 hours. The band intensities of NS1 construct proteins on SDS-PAGE gels were quantified using the Sigma Gel Analysis program and the relative quantities were converted into graphic form. On all the SDS-PAGE gels, lanes 1 show the protein pellet of the gradient (fraction1) and lanes 2 to 13 represent the sequential fractions of the gradient from bottom (highest density) to top (lowest density).

### **2.3.8. Microscopy**

#### **2.3.8.1. Transmission electron microscopy (T.E.M)**

The recombinant NS1 proteins were analysed using T.E.M to determine whether the proteins still formed tubular structures after peptide insertion. AHSV6 NS1, NS1/VP2-40, -150 and NS1/eGFP proteins were prepared for T.E.M analysis as described in section 2.2.13.1. The NS1/VP2-40 and -150 proteins form tubular structures with an average diameter of 33 nm when expressed by recombinant baculoviruses in Sf9 cells (Figure 2.16). This is slightly larger than the AHSV6 NS1 that has an average diameter of 25 nm.

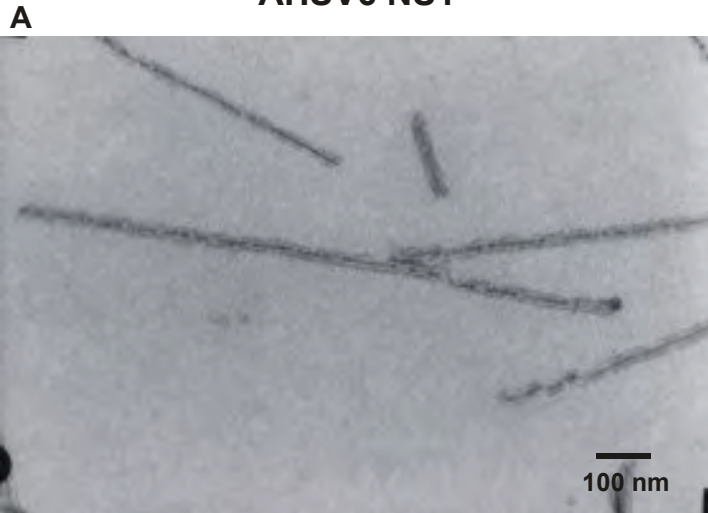
NS1/eGFP proteins expressed in Sf9 cells also formed tubules with an increased diameter of 29 nm (Figure 2.17). The NS1/eGFP-associated tubules are usually observed in regions of dense aggregates and it is not clear if these represent NS1/eGFP aggregates (Figure 2.17). The NS1/eGFP tubules have an internal structure similar to that of wild type NS1 and for both these types of tubules an internal darker lumen is visible that corresponds to the hollow core of the tubule. Although tubular, the structures formed by NS1/VP2-150 and NS1/eGFP do not have a clearly visible internal structure and an internal lumen was not observed. All the proteins formed tubules with varying lengths up to 4  $\mu$ m.

As indicated by red arrows in Figure 2.16(C) baculovirus particles were present in all the samples analysed. These tubules can be distinguished from NS1 tubules based on their larger diameter (50 nm) and the difference in internal structure.

#### **2.3.8.2. Fluorescence microscopy**

NS1/eGFP proteins were prepared for fluorescence microscopy as described in section 2.2.14. Monolayers of uninfected S9 cells were also observed under UV light and served as negative controls to demonstrate that fluorescence can only be attributed to the presence of NS1/eGFP recombinant baculovirus expression in Sf9 cells (Figure 2.18). The cells infected with recombinant baculoviruses expressing NS1/eGFP proteins fluoresces bright green under UV light.

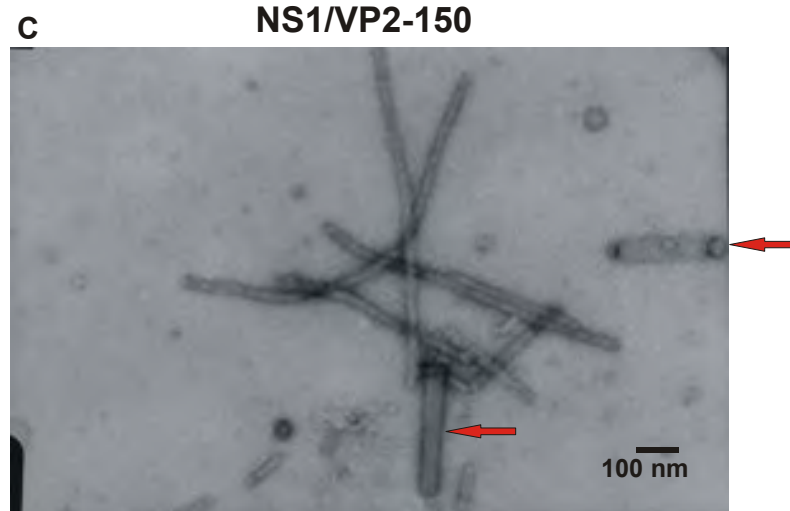
### AHSV6 NS1



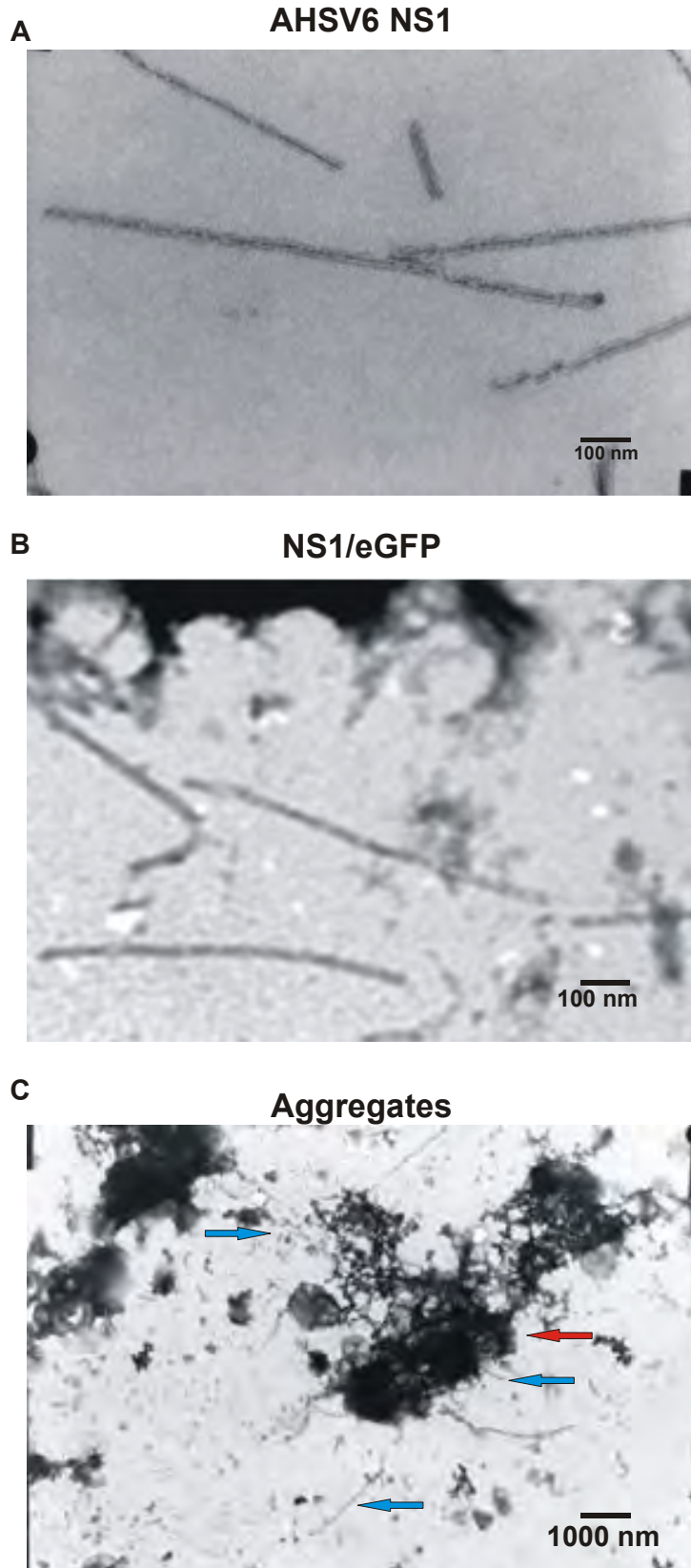
### NS1/VP2-40



### NS1/VP2-150



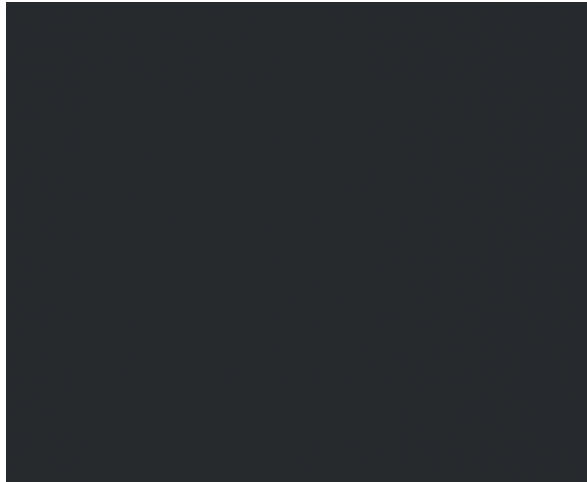
**Figure 2.16.** Negative contrast electron micrographs of negatively stained AHSV6 NS1 tubules (A), recombinant NS1/VP2-40 (B), and NS1/VP2-150 (C) tubules. The baculovirus-expressed NS1 tubules were sucrose gradient purified and stained with 2% uranyl acetate. Contaminating baculovirus tubules can be distinguished from NS1 tubules based on diameter and absence of internal structure and are indicated by red arrows. Bar markers represent 100 nm.



**Figure 2.17.** Negative contrast electron micrographs of negatively stained AHSV6 NS1 tubules (A), recombinant NS1/eGFP (B) tubules, and NS1/eGFP-associated aggregates (C). The baculovirus-expressed NS1 tubules were sucrose gradient purified and stained with 2% uranyl acetate. The red arrow indicates NS1/eGFP aggregates and the blue arrows indicate visible tubules in and around the protein aggregate.

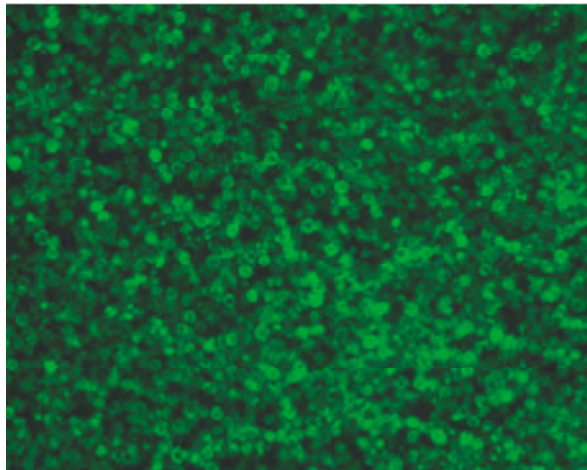
**A**

**Uninfected**



**B**

**NS1/eGFP under UV**



**C**

**NS1/eGFP under Halogen**

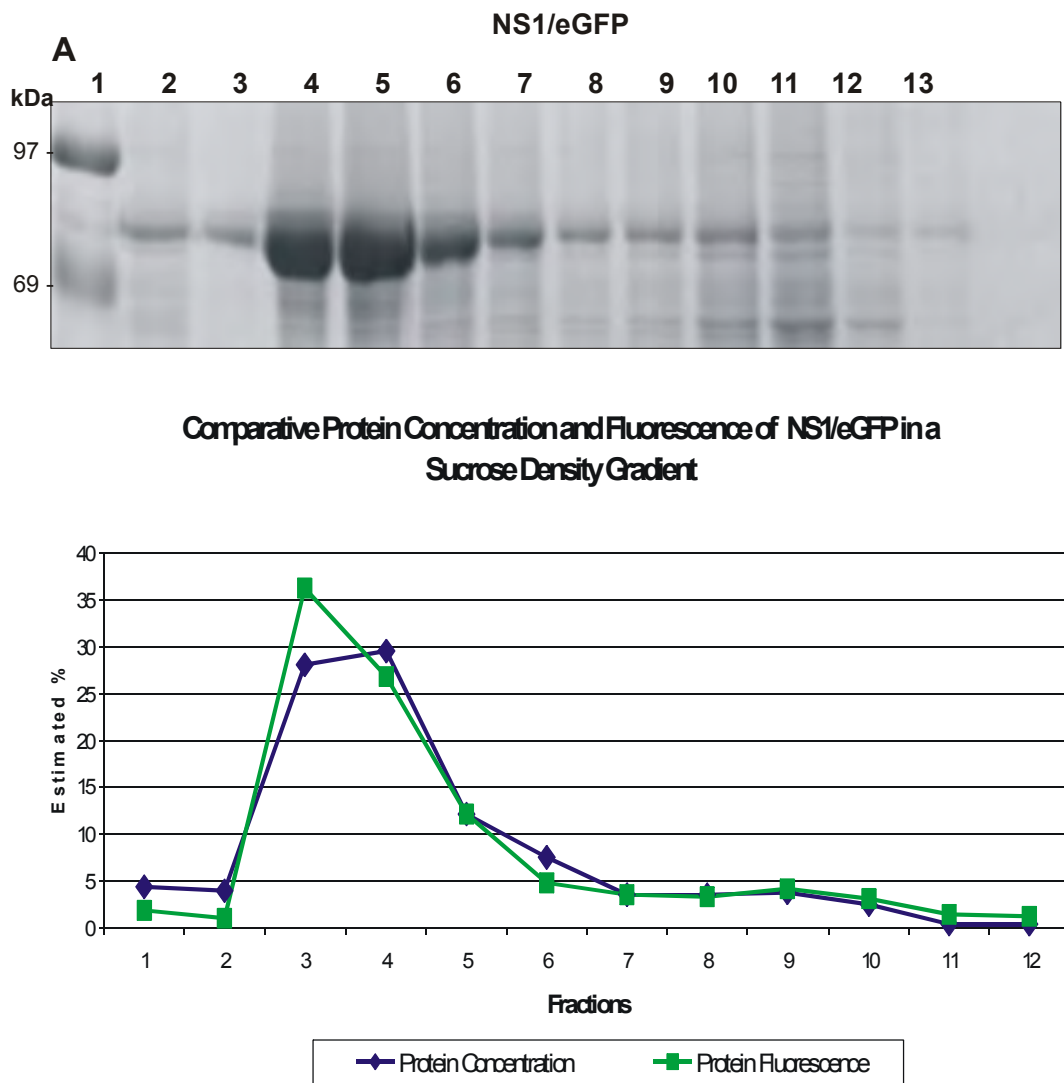


**Figure 2.18.** Fluorescence microscope images of Sf9 cells infected with recombinant NS1/eGFP virus (B) and uninfected Sf9 cells (A). Figure C shows a halogen light microscope image of Sf9 cells infected with recombinant NS1/ eGFP virus.



### 2.3.9. Fluorescence Measurement of NS1/ eGFP Protein Samples

NS1/eGFP proteins were purified as described in section 2.2.11. The proteins were separated on a sucrose gradient by centrifugation at 40 000 rpm for 18 hours at 4°C after which the proteins were fractionated into 400 ul fractions analyzed by both SDS PAGE analysis and by measuring the fluorescence intensity for each fraction (Figure 2.19). Values for both are shown as a percentage of the total protein concentration or fluorescence in the gradient. From the graph (Figure 2.19) it can be seen that there is a direct correlation between the position of NS1/eGFP protein and fluorescence in the sucrose gradient



**Figure 2.19.** Comparative distribution of estimated percentage fluorescence and estimated percentage NS1/eGFP separated on a 50 to 70% sucrose gradient at 40 000 rpm for 18 hours. Lane 1 represents a protein size marker, lanes 2 to 13 represent the gradient fractions, where lane 2 corresponds to fraction 1 (pellet) and lane 13 corresponds to fraction 12 (top of gradient).

### 2.3.10. Storage Stability Studies

It is important to investigate the effect of possible different storage conditions on the structure of chimeric NS1 proteins if these proteins are to be used as subunit vaccines. The different chimeric NS1 proteins were expressed on large-scale and prepared and purified as discussed in section 2.2.12. The chimeric proteins were then subjected to the different temperature conditions at which a vaccine preparation might be stored. These include freeze-drying, flash-freezing in liquid nitrogen (N<sub>2</sub>) and freezing (-70°C).

To limit potential effects of prolonged exposure to 4°C while centrifuging, the treated proteins were separated on sucrose density gradient at 4°C for 2 hours instead of 18 hours. Due to the shorter centrifugation time the proteins of interest were not separated based solely on density but also based on size, as discussed in section 2.3.7. Results from these sucrose gradients will differentiate between particles of high density that differ in physical size and give a more accurate indication of the effect of the specific treatment on protein structure in terms of tubule formation. Untreated controls of AHSV6 NS1 and chimeric NS1 protein constructs were separated on sucrose density gradient under the same conditions as the treated samples and these controls were then compared to the distribution of the treated sample.

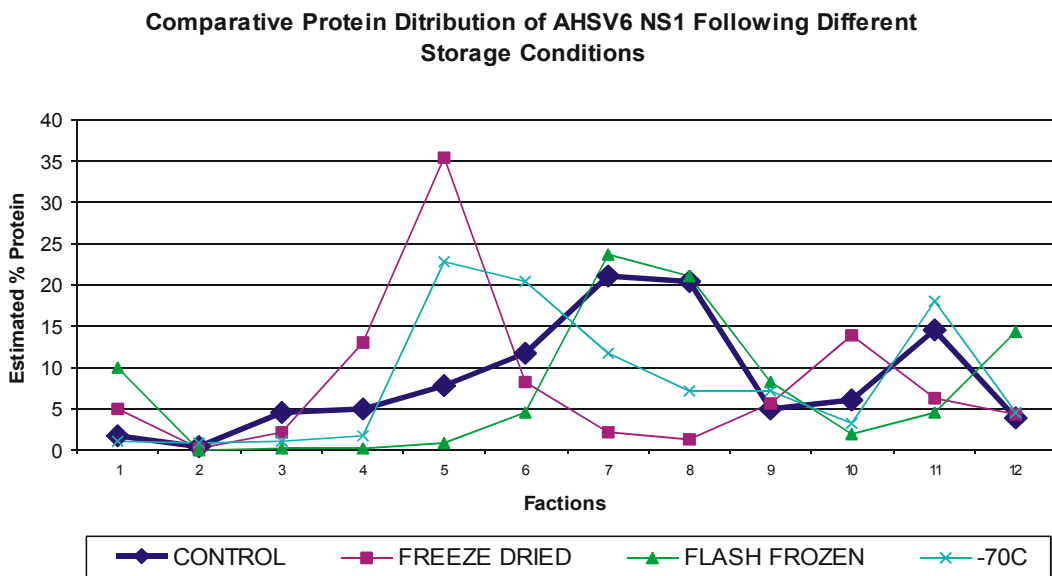
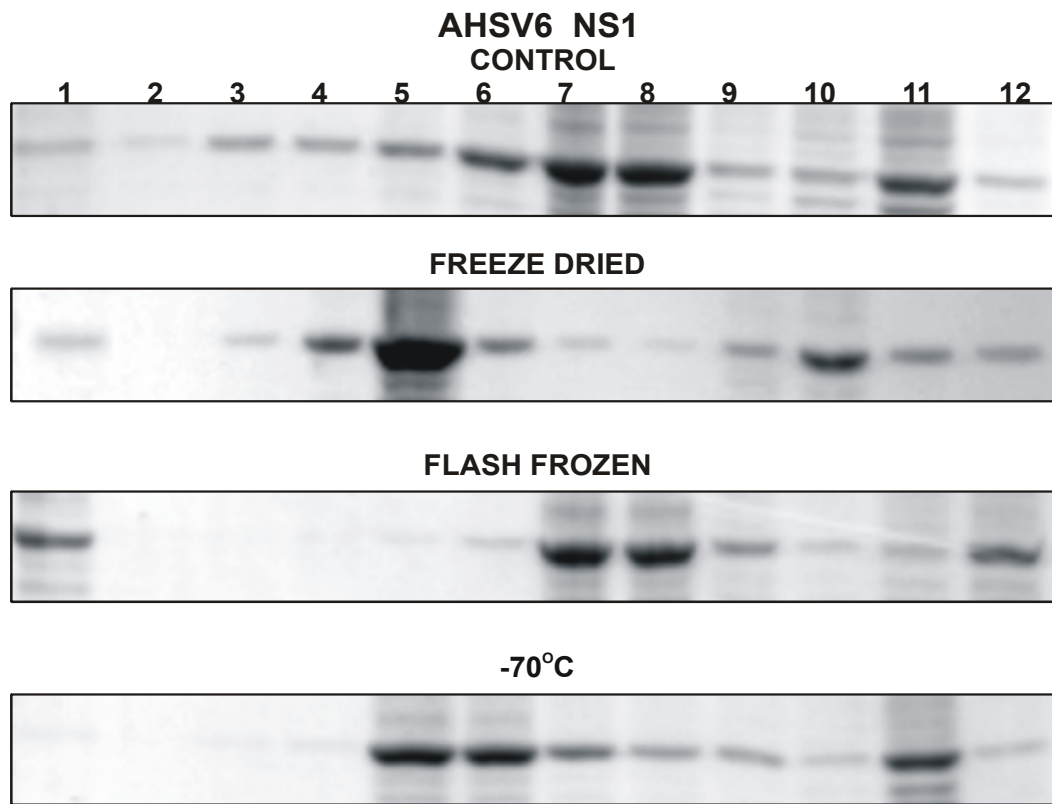
The effect of the different storage conditions on the sucrose gradient distribution of wild type NS1 can be seen in Figure 2.20. The largest proportion of the untreated protein sediment in the bottom two-thirds of the sucrose gradient with the highest concentration of protein in fractions 6 to 8. The second peak is observed at the top of gradient in fraction 11. As previously discussed this fraction probably represents very small NS1 oligomers and tubule subunits. The storage methods do not have a big effect on the distribution of the NS1 proteins in the sucrose gradients. As for the untreated control, the majority of the proteins occur in the bottom two-thirds of the gradient and a low proportion of protein is still present in top part of the fraction. Freeze drying and storage at -70°C appear to result in the NS1 tubules to sediment in sucrose gradient fractions 5-6 rather than 7-8. The change in density could be linked to the level of aggregation of NS1 tubules. If so then both freeze drying and -70°C storage could influence the level of aggregation. In contrast to these results the greatest proportion of the flash frozen NS1 proteins sediment in the same fractions as the untreated sample (fractions 7 and 8) showing that this storage condition has

the least effect on AHSV6 NS1 tubules. Overall these results show that the NS1 protein is relatively stable under different storage conditions and the results from the storage stability studies with the chimeric NS1 proteins will give an indication of the effect of the inserts on the stability of the NS1 protein and tubules.

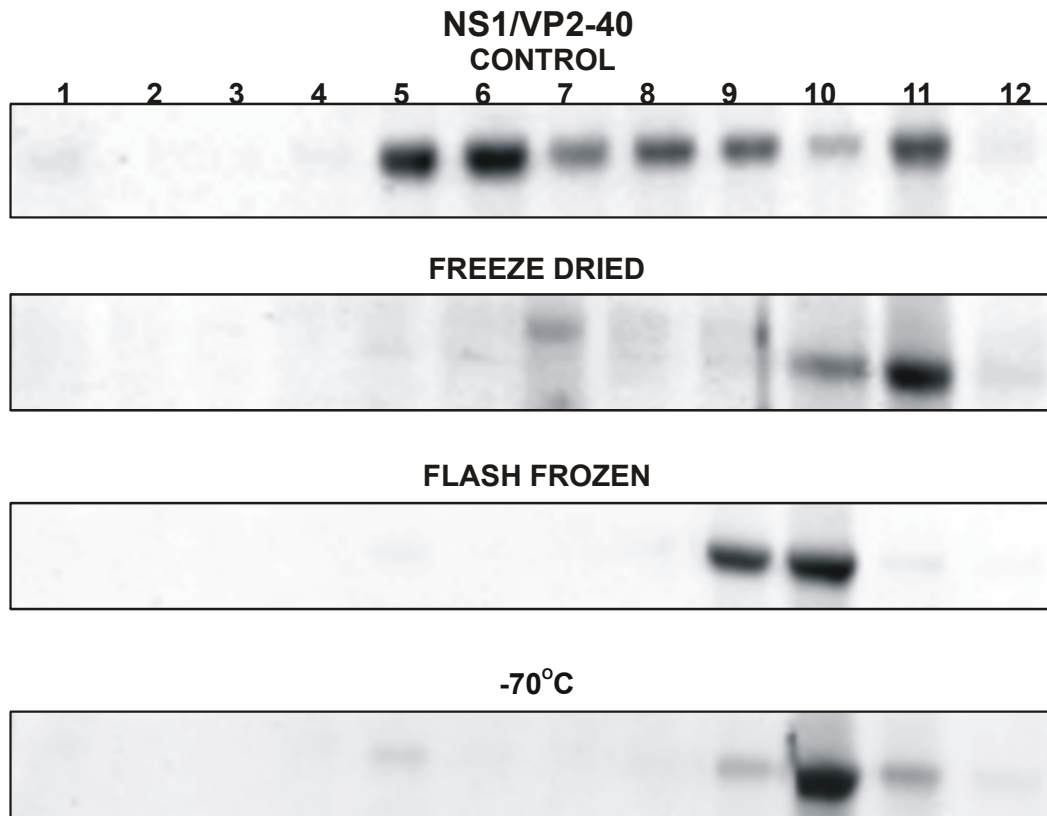
As can be seen from the storage stability study results of NS1/VP2-40 (Figure 2.21) and -150 (Figure 2.22), these proteins have a very similar sucrose gradient protein distribution for all the storage methods. The distribution of the proteins in the sucrose gradients after storage differs greatly from that observed for untreated proteins. For all the storage methods tested, the greatest proportion of the proteins sediment at the top of the gradient. These results indicate that the storage conditions cause the chimeric NS1 tubules to break down into smaller subunits that sediment at the top of the gradient due to their lower sedimentation velocity. The tubular forms of these proteins occur in the higher sucrose density region of the gradient as can be seen for the untreated controls (fractions 5 to 8) and there are no chimeric NS1 proteins present within these regions after storage. From these results it would seem that the inserts destabilize the NS1 tubules and make these structures more vulnerable to temperature treatment, but this is not necessarily negative as will be discussed later on.

The storage stability study results obtained for NS1/eGFP (Figure 2.23) differ greatly from the results obtained for the other two chimeric NS1 proteins (NS1/VP2-40 and -150). Similar to the untreated control, the freeze dried proteins and proteins stored at  $-70^{\circ}\text{C}$  sediment in the bottom third of the gradient showing that the majority of tubule structures are stable during these storage conditions. In contrast to the untreated control and samples stored at  $-70^{\circ}\text{C}$ , the majority of the freeze dried proteins occur in the pellet fraction of the gradient which would seem to indicate that this treatment causes high level aggregation of the NS1/eGFP tubule subunits and/or tubules. Flash freezing appears to reduce tubule size as observed by the proteins sedimenting in fractions closer to the top of the gradient. This could be due to a reduction in the size of the tubules but most likely a reduction in the level of NS1 tubule aggregation. The greatest proportion of flash frozen NS1-eGFP proteins occurs at a higher position in the gradient than the untreated control. The proteins probably represent smaller tubule fragments caused by destabilization of bigger structures that are now present at lower concentrations in fractions 3 to 6. These results show that the NS1/eGFP protein is most stable when stored at  $-70^{\circ}\text{C}$  and that the tubules formed by the

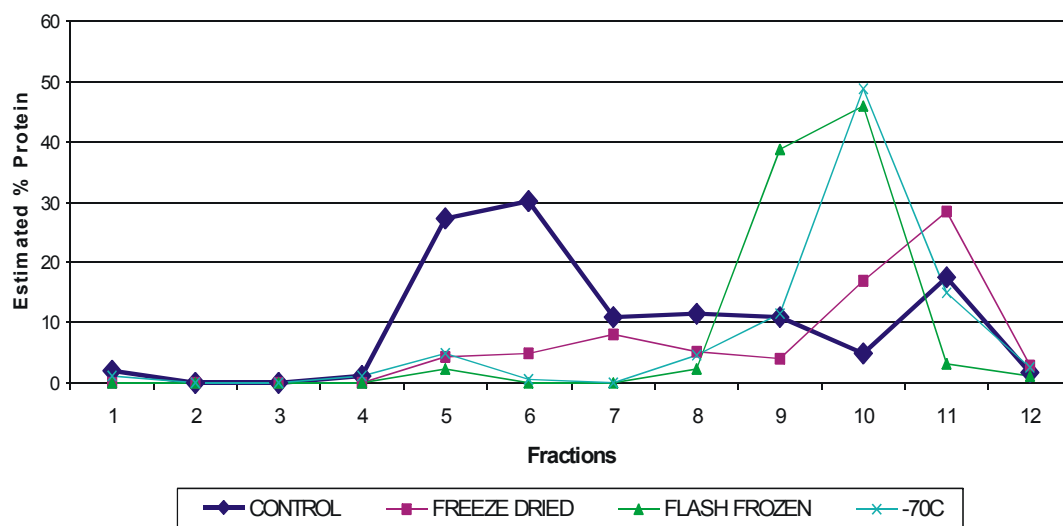
NS1/eGFP protein are highly stable under different storage conditions. This stability can in part be attributed to the highly stable eGFP insert.



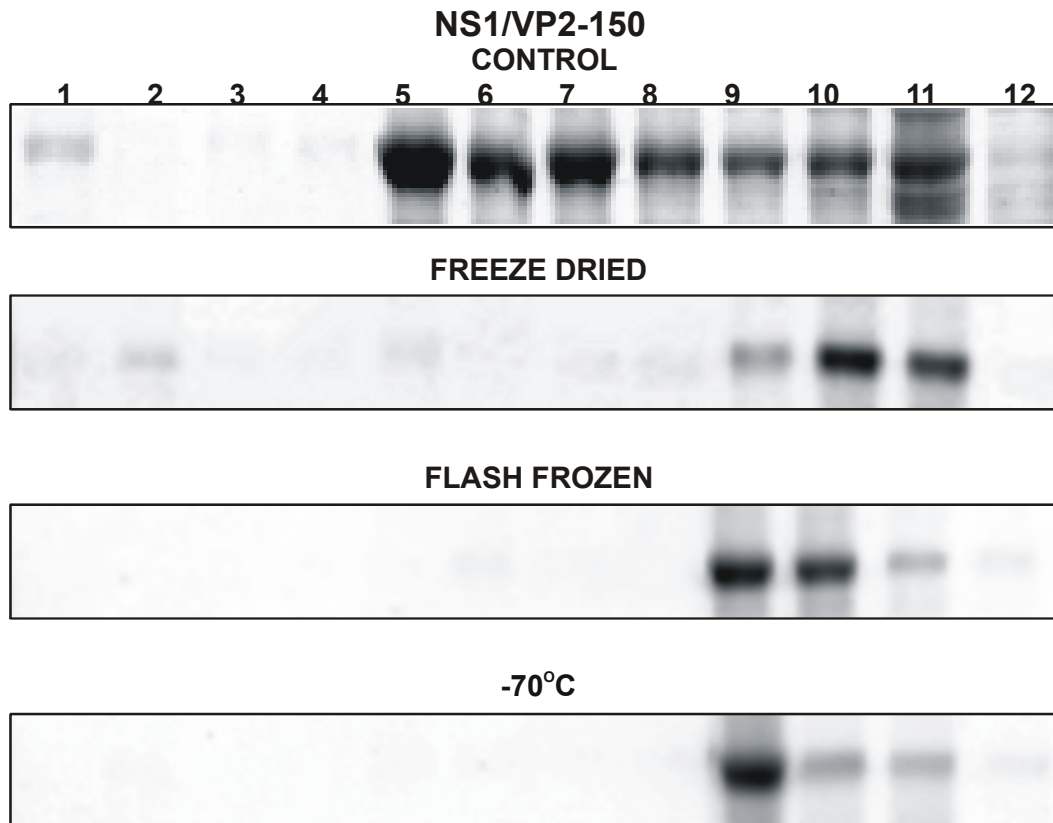
**Figure 2.20.** Comparative sucrose gradient protein distribution of AHSV6 NS1 samples stored at different conditions. Proteins were separated under centrifugation conditions of 40 000 rpm for 2h at 4°C on a 50 to 70% sucrose gradient. The Sigma Gel™ software package was used to quantify estimated band intensities and the relative quantities converted into graphic form. For all the SDS-PAGE gels, lanes 1 show the gradient pellet (fraction 1) and lanes 2 to 12 represent the sequential fractions of the gradient from bottom (highest density) to top (lowest density).



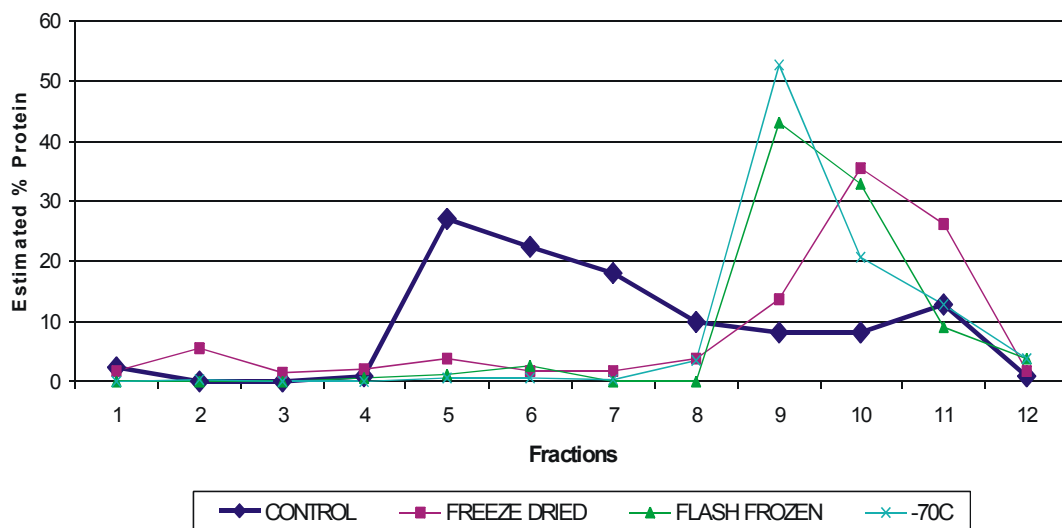
**Comparative Particle Distribution of NS1/VP2-40 on Sucrose Gradient Following Different Storage Conditions**



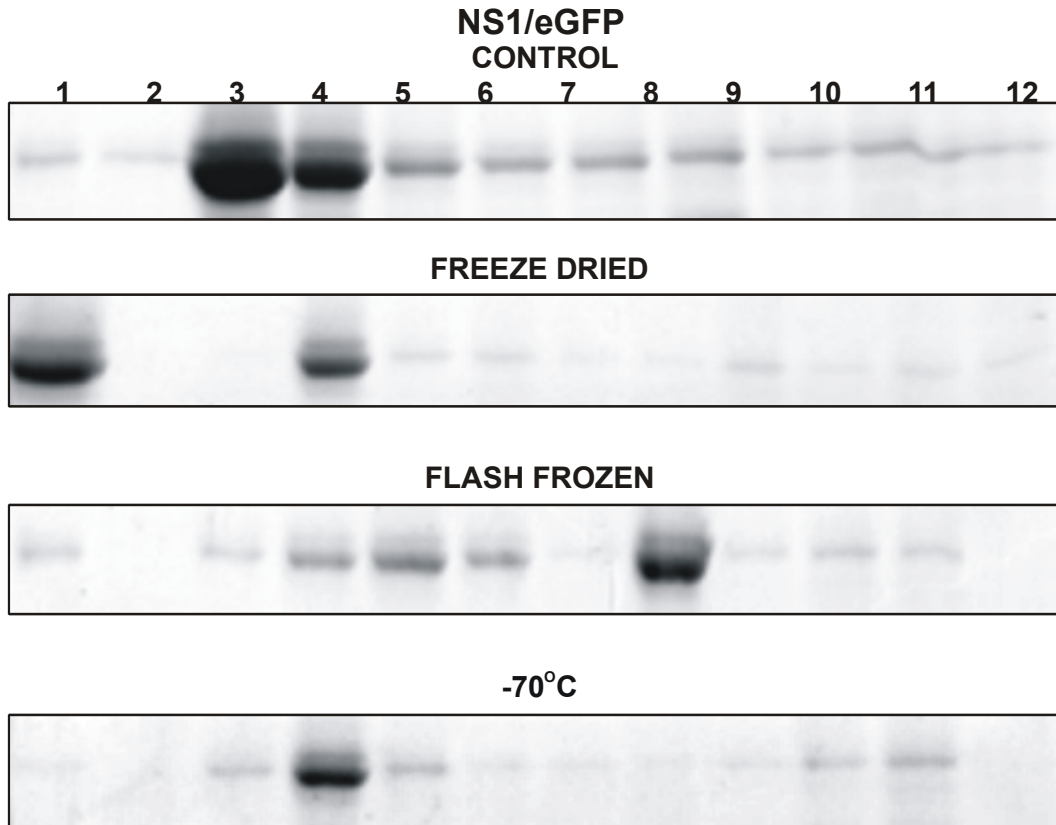
**Figure 2.21.** Comparative sucrose gradient protein distribution of NS1/VP2-40 samples stored at different conditions. Proteins were separated under centrifugation conditions of 40 000 rpm for 2h at 4°C on a 50 to 70% sucrose gradient. The Sigma Gel™ software package was used to quantify estimated band intensities and the relative quantities converted into graphic form. For all the SDS-PAGE gels, lanes 1 show the gradient pellet (fraction 1) and lanes 2 to 12 represent the sequential fractions of the gradient from bottom (highest density) to top (lowest density).



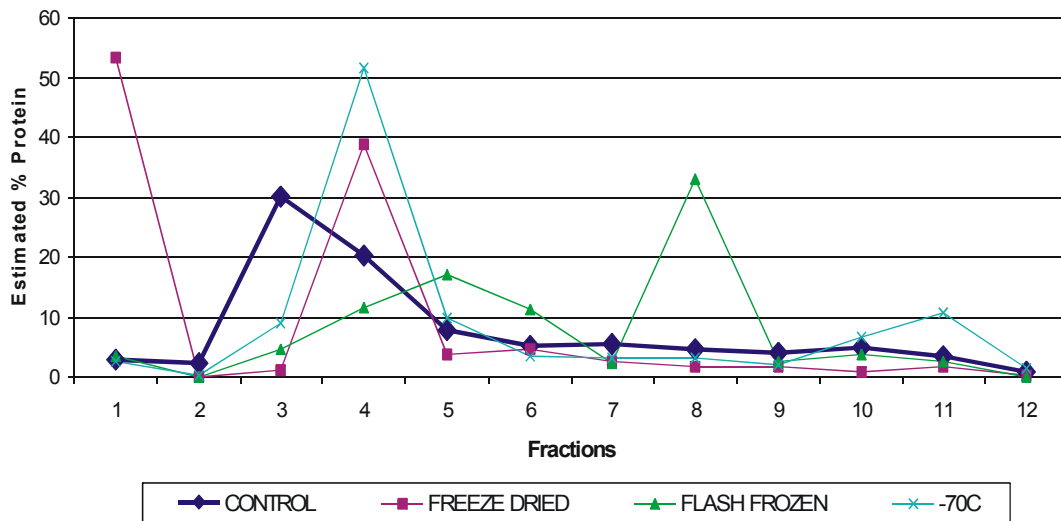
**Comparative Protein Distribution of NS1/VP2-150 on Sucrose Gradient  
Following Different Storage Conditions**



**Figure 2.22.** Comparative sucrose gradient protein distribution of NS1/VP2-150 samples stored at different conditions. Proteins were separated under centrifugation conditions of 40 000 rpm for 2h at 4°C on a 50 to 70% sucrose gradient. The Sigma Gel™ software package was used to quantify estimated band intensities and the relative quantities converted into graphic form. For all the SDS-PAGE gels, lanes 1 show the gradient pellet (fraction 1) and lanes 2 to 12 represent the sequential fractions of the gradient from bottom (highest density) to top (lowest density).



**Comparative Protein Distribution of NS1/eGFP on Sucrose Gradient  
Following Different Storage Conditions**



**Figure 2.23.** Comparative sucrose gradient protein distribution of NS1/eGFP samples stored at different conditions. Proteins were separated under centrifugation conditions of 40 000 rpm for 2h at 4°C on a 50 to 70% sucrose gradient. The Sigma Gel™ software package was used to quantify estimated band intensities and the relative quantities converted into graphic form. For all the SDS-PAGE gels, lanes 1 show the gradient pellet (fraction 1) and lanes 2 to 12 represent the sequential fractions of the gradient from bottom (highest density) to top (lowest density).

### 2.3.11. Western Blot Analysis

This preliminary immunological study had two main aims. The first aim was to determine if the NS1 protein is able to present the inserted peptides to the immune system (immunogenicity). For this purpose, antisera were generated against baculovirus-expressed AHSV6 NS1, NS1-VP2/40, NS1-VP2/150 and NS1-eGFP in Guinea pigs, as described in section 2.2.15. These sera were then tested to establish if the generated serum antibodies can recognize the native NS1 protein, the chimeric protein used to make the specific antiserum and the inserts presented in either the NS1 protein or AHSV VP7 (Riley, 2003); Mizrachi, unpublished results) using Western blotting. Secondly, this study aimed to evaluate if inserts presented on the NS1 vector are still able to bind antibodies produced against the native protein or protein presented in the AHSV VP7 carrier protein (antigenicity). For this aim insert-specific antisera were either obtained commercially or sourced from related studies where antiserum was generated to the eGFP protein inserted into the AHSV9 VP7 protein (Mizrachi, unpublished results). These sera were then tested using Western blotting as discussed in section 2.2.16.

For the first set of studies aimed to determine the immunogenicity of the chimeric NS1 proteins, antisera were generated in guinea pigs as discussed in section 2.2.15. Serum generated against the native AHSV NS1 protein was included as control in this study to determine the immunogenicity of the carrier without any insert. In the Western blot analysis the antisera were tested against a range of different protein extracts that included:

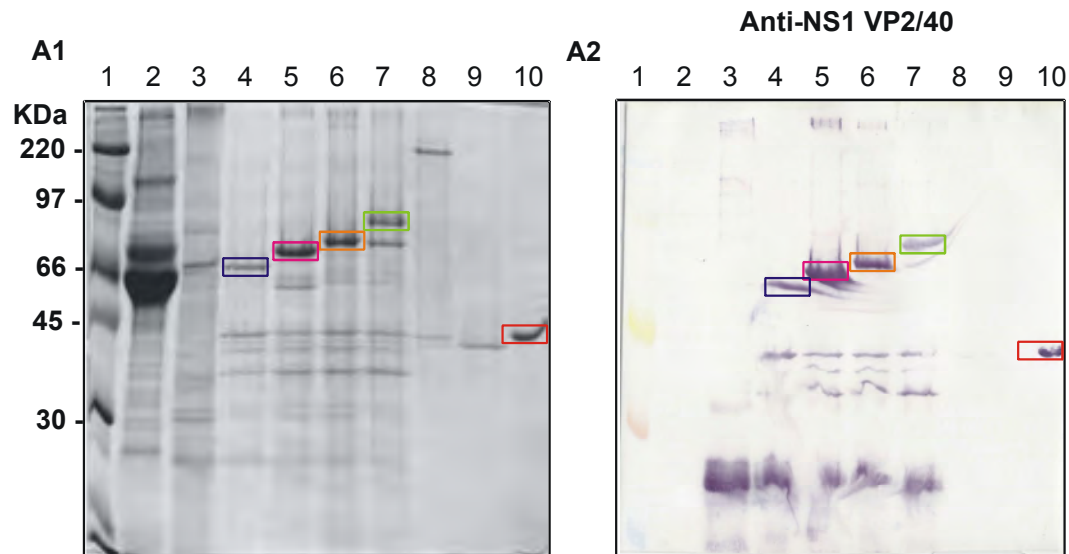
- Uninfected Sf9 cell protein extracts (moc).
- Wild type baculovirus infected Sf9 cell protein extract.
- Wild type AHSV NS1
- NS1/VP2-40
- NS1/VP2-150
- NS1/eGFP
- AHSV9 VP7. This protein served as a negative control.
- VP7-144. The VP7-144 vector protein is wild type AHSV VP7 modified by the insertion of 6 amino acids between amino acid 144 and 145 to allow the insertion of DNA in the MCS of the VP7-144 vector (Riley, 2003). Served as a negative control.
- VP7-144-VP2. This protein has a region of AHSV9 VP2 (amino acid 377 to 401) inserted into the VP7-144 vector protein (Riley, 2003). The VP2 insert



corresponds to the AHSV9 VP2 region that is inserted into the NS1/VP2-40 and -150 proteins.

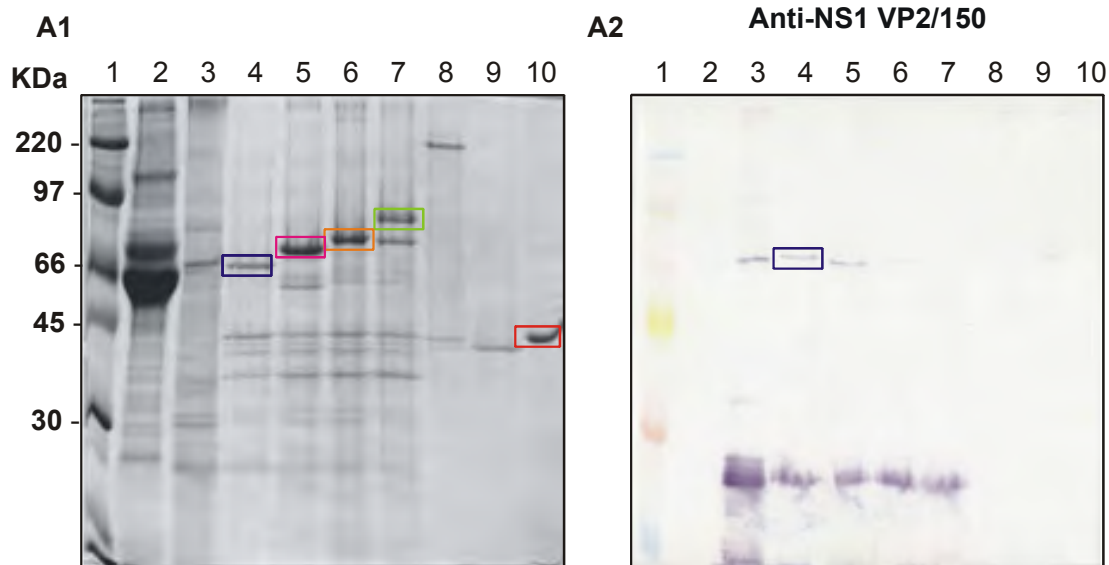
The prepared antiserum from guinea pigs was pooled and used as the primary antibody at a one in thirty dilution. The guinea pig serum generated against AHSV6 NS1 was not able to react with any of the NS1 constructs, including the original baculovirus-expressed AHSV6 NS1 protein that was used for antiserum generation (results not shown). The serum reacted with Sf9 cell-specific proteins and wild type baculovirus proteins, confirming that the Western blot procedure worked. The reason for the inability of NS1 to elicit an immune response is unclear, but could be due to either low inherent immunogenicity of the protein.

As can be seen from Figure 2.24 the serum generated against baculovirus-expressed NS1-VP2/40 recognized all the different NS1 fusion proteins as well as wild type NS1. The reaction against wild type NS1 and NS1 with the eGFP insert is however weaker, suggesting that this was only due to NS1 specific antibodies. The reaction against the fusion NS1 that also contain the VP2 insert appears to be stronger, suggesting an insert specific immune reaction. That this is indeed the case is confirmed by the ability of the serum to recognize an AHSV9 VP2 insert cloned into the 144 site of VP7 (Riley, 2003). This reaction is insert-specific because there is no reaction to VP7-144 without the VP2 insert. This result confirms that the antibodies generated against the NS1-VP2/40 construct are not exclusively against the NS1 vector, but that a very good reaction is elicited against the peptide insert.



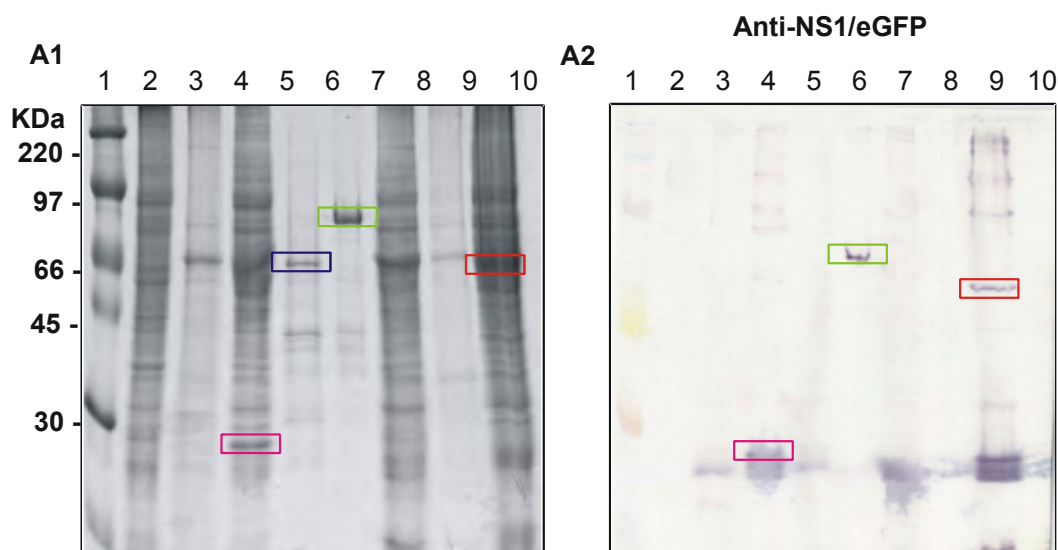
**Figure 2.24.** Western blot analysis showing the **anti-NS1/VP2-40** serum probed to chimeric NS1 and AHSV Vp7 proteins. (A1) Extracts obtained from uninfected Sf9 cells (lane 2) and Sf9 cells infected with baculovirus expressing wild type baculovirus proteins (lane 3), AHSV6 NS1 (lane 4), NS1/VP2-40 (lane 5), NS1/VP2-150 (lane 6), NS1/eGFP (lane 7), AHSV9 VP7 (lane 8), VP7-144 vector (lane 9) and VP7-144/VP2 (lane 10) were resolved on 12% SDS-PAGE and stained with Coomassie brilliant blue. Rainbow marker was used as size reference (lane 1). The resolved products were electroblotted onto nitrocellulose membrane (A2) and reacted with guinea pig anti-NS1/VP2-40 antiserum. Positions of markers (in kilodaltons) are indicated. The position of AHSV6 NS1 is indicated in blue NS1/VP2-150 in orange, NS1/eGFP in green and VP7-144/VP2 in red.

The serum generated against the NS1-VP2/150 construct did not react with any of the NS1 constructs and showed only low-level reaction with the wild type NS1 protein (Figure 2.25). The serum was also unable to react with the AHSV9 VP2 insert presented in the VP7-144 system (VP7-144-VP2) (Riley, 2003). This result is not totally unexpected as transmission electron microscopy studies of the NS1-VP2/150 protein showed that the resulting tubules, though still similar to native AHSV tubules, exhibit structural differences such as loss of discernable internal structure and change in diameter. It is possible that the large size of the insert distorts the NS1 structure in such a way that the peptide insert is masked and not accessible to the immune system.



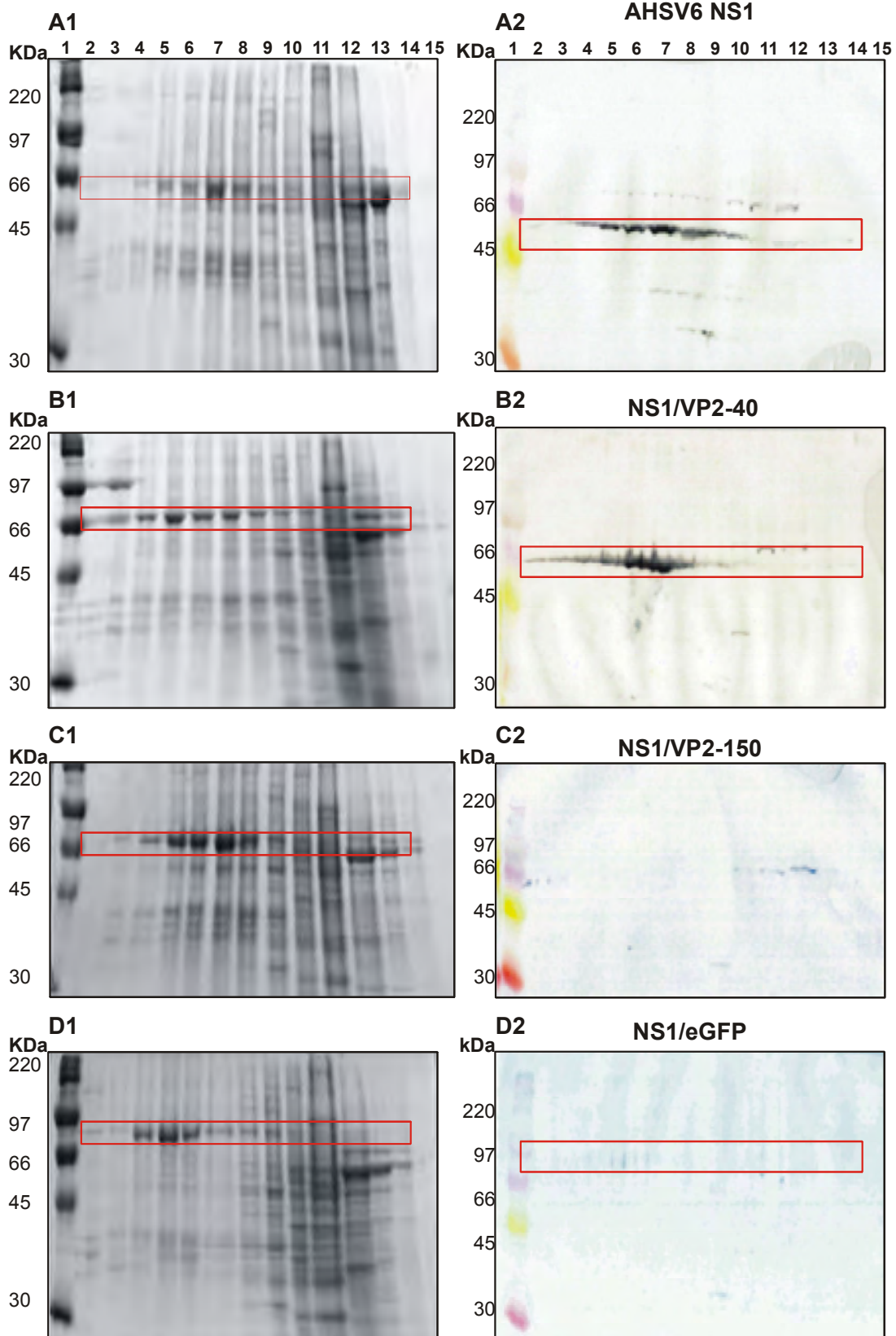
**Figure 2.25.** Western blot analysis showing the anti-NS1/VP2-150 serum probed to chimeric NS1 and AHSV VP7 proteins . (A1) Extracts obtained from uninfected Sf9 cells (lane 2) and Sf9 cells infected with baculovirus expressing wild type baculovirus proteins (lane 3), AHSV6 NS1 (lane 4), NS1/VP2-40 (lane 5), NS1/VP2-150 (lane 6), NS1/eGFP (lane 7), AHSV9 VP7 (lane 8), VP7-144 vector (lane 9) and VP7-144/VP2 (lane 10) were separated by 12% SDS-PAGE and stained with Coomassie brilliant blue. Rainbow marker was used as size reference (lane 1). The resolved products were electroblotted onto nitrocellulose membrane (A2) and reacted with guinea pig anti-NS1/VP2-150 antiserum. Positions of markers (in kilodaltons) are indicated. The position of AHSV6 NS1 is indicated in blue, NS1/VP2-150 in orange, NS1/eGFP green and VP7-144/VP2 red.

Serum generated against the NS1/eGFP was probed to baculovirus-expressed eGFP (positive control), AHSV6 NS1, NS1/eGFP, VP7-177-eGFP (Mizrachi, unpublished results), AHSV NS2 (negative control) and VP7-177-NS2 (negative control). The VP7-177-eGFP protein consists of the eGFP marker protein inserted into site 177 on the AHSV VP7 top domain. Serum obtained from guinea pigs immunized with the NS1-eGFP construct, reacted with the eGFP protein, NS1-eGFP protein and the VP7 protein with the eGFP insert. The serum did not react with the wild type NS1 protein or the VP7 and NS2 controls (figure 2.26). This result indicates that the eGFP presented in the NS1 protein is able to elicit antibodies and that these antibodies can bind to the eGFP protein on its own or presented in a presentation system (VP7-177-eGFP). The antiserum does not react with the wild type NS1 protein, indicating the antibodies in the serum were generated against the eGFP insert rather than the NS1 carrier protein.



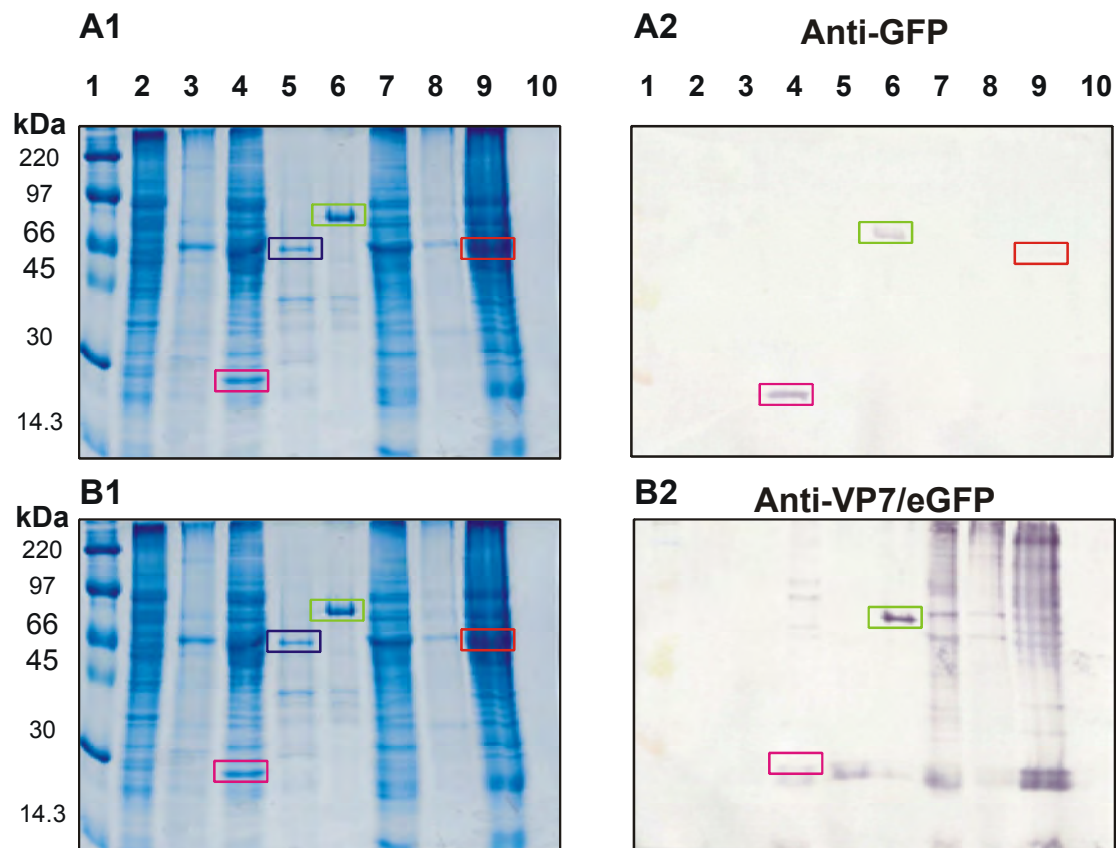
**Figure 2.26.** Western blot analysis showing the **anti-NS1/eGFP** serum targeted to chimeric NS1 and chimeric VP7/eGFP proteins. (A1) Extracts obtained from uninfected Sf9 cells (lane 2) and Sf9 cells infected with baculovirus expressing wild type baculovirus proteins (lane 3), eGFP (lane 4), NS1 (lane 5), NS1/eGFP (lane 6), AHSV NS2 (lane 7), VP7/NS2 (lane 8), and VP7/eGFP (lane 9) were separated by 12% SDS-PAGE and stained with Coomassie brilliant blue. Rainbow marker was used as size reference (lane 1). The resolved products were electroblotted onto nitrocellulose membrane (A2) and reacted with guinea pig anti-NS1/eGFP antiserum. Positions of markers (in kilodaltons) are indicated. The position of the eGFP protein is indicated in purple, AHSV6 NS1 in blue, NS1/eGFP in green and VP7/eGFP in red.

The above results were confirmed by Western blot analysis using AHSV3-specific serum to probe the proteins of all the chimeric NS1 constructs as well as native NS1 (Figure 2.27). Total sucrose gradients were specifically used to prove that results were not biased due to the use of specific gradient fractions. The AHSV3-specific antiserum reacted with both AHSV6 NS1 and NS1-VP2/40 proteins as can be seen in Figure 2.27. The serum showed no reaction with either NS1-VP2/150 or NS1-eGFP, which supports results from Western blot analysis with anti-NS1-VP2/150 serum. It is possible that that NS1-VP2/150 protein structure is altered to such an extent that it is no longer recognized by antibodies against native NS1.



**Figure 2.27.** 10 % polyacrylamide gels (A1 to D1) and western blot analysis showing the anti-AHSV3 serum probed to 50-70% sucrose gradient fractions of baculovirus expressed AHSV6 NS1 (A2), NS1-VP2/40 (B2), NS1-VP2/150 (C2) and NS1-eGFP (D2). In all the figures lane 1 contains the protein size marker, lanes 2-14 show the sucrose gradient fractions where lane 2 corresponds to the gradient pellet (bottom of gradient) and lane 14 contains the top fraction of the gradient (lowest density). Red blocks indicate the positions of AHSV6 NS1 and chimeric NS1-constructs

The antigenicity of inserts presented on the NS1 protein was tested by performing Western blot analysis with antisera generated against the eGFP protein in rabbits (Sigma-Aldrich) and eGFP presented in AHSV VP7 (Mizrachi, unpublished). The sera were reacted with baculovirus-expressed eGFP protein, AHSV6 NS1 (negative control), NS1-eGFP, AHSV NS2 (negative control), VP7-NS2 (negative control) and VP7/eGFP (positive control). Western blot analysis performed with antiserum specific for GFP (Sigma-Aldrich) showed that eGFP is recognized in context of the NS1 presentation system (Figure 2.28). The serum generated to VP7/eGFP also reacted with the NS1/eGFP protein. These results indicate that peptides (eGFP) presented in the NS1 protein are recognized by antibodies generated by native eGFP confirming the insert retain their antigenicity.



**Figure 2.28.** Western blot analysis showing **anti-eGFP**(A2) and **anti-VP/eGFP** (B2) sera probed to chimeric NS1 and chimeric VP7/eGFP proteins . (A1) Extracts obtained from uninfected Sf9 cells (lane 2) and Sf9 cells infected with baculovirus expressing wild type baculovirus proteins (lane 3), eGFP (lane 4), NS1 (lane 5), NS1/eGFP (lane 6), AHSV NS2 (lane 7), VP7/NS2 (lane 8), and VP7/eGFP (lane 9) were separated by 12% SDS-PAGE and stained with Coomassie brilliant blue (A1 and B1). Rainbow marker was used as size reference (lane 1). The resolved products were electroblotted onto nitrocellulose membrane (A2; B2) and reacted with guinea pig anti-NS1/eGFP or rabbit anti-GFP antiserum. Positions of markers (in kilodaltons) are indicated. The position of the eGFP protein is indicated in purple, AHSV6 NS1 in blue, NS1/eGFP in green and VP7/eGFP in red.

## 2.4 DISCUSSION

Gould *et al.* (1994) first suggested the insertion of foreign epitopes into sites displayed on the surface of the NS1 protein (Gould *et al.*, 1994). This suggestion was based on the finding by Eaton *et al.* (1988) that the epitopes bound by a panel of monoclonal NS1 antibodies are located on the surface of the macromolecular NS1 tubule (Eaton *et al.*, 1988). Peptides cloned into these hydrophilic domains in the NS1 protein monomers should be displayed theoretically as multiple copies on the surface of the tubular macromolecules, augmenting immune response to epitopes (Ghosh *et al.*, 2002a). This system could potentially form the basis for the construction of vaccines against infectious organisms with known and defined neutralising epitopes. Various studies have shown that that these recombinant tubules can be used a carrier system for immunogenic epitopes and induced protective humoral and cell-mediated responses (Ghosh *et al.*, 2002a; Ghosh *et al.*, 2002b; Ghosh *et al.*, 2002c; Larke *et al.*, 2005). The BTV NS1 tubules can accommodate proteins attached to the C-terminal as large as 527 amino acids without losing their structure (Larke *et al.*, 2005). The primary objective of this study was to evaluate the potential of AHSV NS1 tubules as a peptide carrier system for inserts of a fairly wide size range.

The first aim of the study was to identify a suitable site for peptide insertion in the NS1 protein that would display inserts on the surface of the tubule structure. The carboxy terminus region of the AHSV NS1 protein was targeted for peptide insertion as studies on BTV NS1 suggest that the carboxy terminus of NS1 is exposed on the surface of tubules. Firstly, the primary antigenic site has been localized to the carboxy terminus and monoclonal antibodies raised against linear epitopes corresponding to the carboxy terminus react specifically with intact tubules (Monastyrskaya *et al.*, 1995). Secondly, the addition of extra peptide sequences and proteins to the carboxy terminus of BTV NS1 has no effect on tubule formation (Ghosh *et al.*, 2002a; Ghosh *et al.*, 2002b; Ghosh *et al.*, 2002c; Mikhailov *et al.*, 1996). The choice of an insertion site in an internal position of the C-terminal was based on hydrophilicity and antigenicity comparisons of the AHSV6 NS1 protein. Based on the comparison of hydrophilicity (Hopp, Woods, 1981; Hopp, Woods, 1983) and antigenicity (Welling *et al.*, 1985) profiles, an overlapping region of antigenicity and hydrophilicity was identified within the C-terminal region of the protein (Figure 2.2). This region corresponds to amino acid position 470-504 and contains a unique *Xho*I site within the AHSV6 NS1 gene (nucleotide position 1414-1420 of the coding sequence). This region also corresponds well to an identified antigenic epitope

(position 474 – 502) in BTV NS1 (Du plessis *et al.*, 1995) and a region recognized by a BTV NS1 monoclonal antibody (Eaton *et al.*, 1988; Monastyrskaya *et al.*, 1995) Foreign peptides can be cloned into this native restriction enzyme site, without compromising NS1 structure stability due to the insertion of additional restriction enzyme sites to accommodate cloning of inserts and hence additional amino acids. Structure stability of chimeric NS1 proteins will be a function of the size and character of peptides inserted into the native *XhoI* site.

Three peptides within a size range of 40 to 244 amino acids were inserted into the identified *XhoI* site in the NS1 gene. Two overlapping regions of AHSV9 VP2 of 40 and 150 amino acids were selected based on the previously identified antigenic regions of AHSV and BTV VP2 as summarized in Table 2.1. The full length enhanced green fluorescent protein (240 aa), excluding the stop codon, was also inserted into the *XhoI* site of AHSV6 NS1. Enhanced eGFP is a red-shifted variant of wild-type GFP (Chalfie *et al.*, 1994; Inouye, Tsuji, 1994; Prasher *et al.*, 1992). The insertion of the 121 bp, 450 bp and 720 bp (excluding additional *XhoI* site) peptides in the correct reading frame and orientation (non-directional cloning) were confirmed by sequencing.

The primary research objective of this study was to investigate the effect of the insertion of differently sized peptides have on particle formation. Complete abolishment of tubule assembly is undesirable as particulate structure is used as presentation platform for foreign peptides. Sucrose density gradients and transmission electron microscopy were used to investigate the ability of the recombinant NS1 proteins to form stable particulate structures that resemble wild type AHSV NS1 tubules.

Transmission electron micrographs of negatively stained recombinant NS1 proteins indicated that these proteins all formed tubules similar, but not identical to those formed by wild type (AHSV6) NS1. The tubules formed by NS1/VP2-40 and -150 have an increased diameter of 33 nm and the NS1/eGFP proteins also form tubules with an increased diameter of 29 nm. The increased diameters of the chimeric tubules when compared to the 25 nm diameter of wild type tubules can be explained in terms of the size of the inserts as well as the physico chemical nature of the inserts a discussed in section 2.3.5. The hydrophilic nature of the VP2-derived inserts creates a larger hydrophilic region in the NS1 protein (section 2.3.5) that could lead to the protein being less densely packaged due to steric hindrance. This decreased



density could cause the increased diameter of the tubular structures carrying VP2 inserts. In the case of the chimeric NS1/eGFP, the insert is hydrophobic, but the tubules still have an increased diameter. This can perhaps be explained in terms of the barrel-like nature of the eGFP protein which causes hydrophilic regions of the protein to be exposed on the surface of the tubule while masking the hydrophobic regions of the protein within the tubular structure, hence creating a quasi hydrophilic protein that is displayed on the surface of the NS1 protein in very similar fashion to the hydrophilic VP2 inserts. This also partly explains why the NS1/eGFP tubule diameter is smaller than the other two tubules that contain smaller inserts. Although the eGFP insert is considerably larger in terms of amino acid size it is packaged more densely into a tight barrel-like structure. All the tubules varied in length and tubules of up to 4  $\mu\text{m}$  in length were observed. The NS1/VP2-150 and NS1/eGFP tubules do not display a central lumen as observed for wild type NS1 and NS1/VP2-40. The variation in tubule length could be attributed to different stages of tubule formation or could just be the cause of mechanical tubule shearing during preparation. As far as could be discerned no specific fraction was associated with tubules of a specific average length. Similar studies on BTV NS1 also showed that chimeric tubules carrying peptides have morphologies similar to wild-type NS1 tubules. In contrast to the chimeric NS1 tubules the chimeric BTV tubules did to lose their distinctive internal structure even after insertion of the large eGFP protein. Insertion of a 527 amino acid protein caused only minor disturbances of the tubular internal structure (Larke *et al.*, 2005). These differences in the chimeric tubule structures of AHSV and BTV could be attributed to the fact that BTV tubules are more stable than AHSV and the different positions of the peptide insertion sites (Ghosh *et al.*, 2002a; Ghosh *et al.*, 2002b; Ghosh *et al.*, 2002c; Monastyrskaya *et al.*, 1995).

Sucrose gradient density analysis of baculovirus expressed proteins was utilized not only to purify the different NS1 constructs but also to further analyze the effect of peptide inserts on tubule formation and protein structure. Before commencing with sucrose density gradient analysis of protein samples an experiment was performed with the AHSV6 NS1 protein to determine if proteins are separated based on size or/and density. A comparison of 50 to 70% sucrose gradients of AHSV6 NS1 proteins centrifuged at high velocity (40 000 rpm) for either 2 or 18 hours indicated that the separation of proteins on gradient are not solely based on density but influenced by the physical size of the protein (section 2.3.7).

The effect of inserts on the structure of the chimeric NS1 proteins were analysed by comparison of the sucrose gradient profile of the chimeric proteins to that of AHSV NS1. For all the constructs a typical pattern of multimeric particle distribution was observed. The majority of the recombinant NS1 proteins is concentrated within the bottom two-thirds of the gradient (excluding pellet fraction) and is consistent with tubule formation (Ghosh *et al.*, 2002a; Ghosh *et al.*, 2002b; Ghosh *et al.*, 2002c; Huismans, Els, 1979; Maree, 2000; Maree, Huismans, 1997; Monastyrskaya *et al.*, 1995; Owens *et al.*, 2004; Urakawa, Roy, 1988). The distribution of proteins through all the fractions of the lower third of all the gradients can be attributed to the assembly process of NS1 into tubules. Each of these stages in tubule assembly is associated with a different density. These results support the findings from the TEM studies that showed that the chimeric NS1 proteins are still able to assemble into tubular structures.

The chimeric proteins, NS1/VP2-40 and -150, show a decrease in protein concentration in fractions 1 and fractions 1 to 3, respectively, when compared the sucrose gradient distribution of native AHSV6 NS1 (Figure 2.15). These two proteins do not form the higher density protein particles observed for native NS1. These differences in density sedimentation of the proteins can probably be attributed to the hydrophilic nature of the inserts that could cause a decrease in the density of tubule assembly (as discussed in section 2.3.5), hence less dense particles that reach density equilibrium at a lower density than native NS1 tubules. The decrease in density could cause a protein with an increased physical size due to looser packaging of subunits and this could also influence the ratio of the lumen/ tubule volume. This ratio can in turn influence the overall observed density of the protein as the lumen decreases the overall density of the tubule. Transmission electron micrographs (Figure 2.16) confirm that the recombinant proteins form slightly thicker tubules when compared to wild type NS1 tubules (5 nm). The NS1/eGFP protein that sediments at a slightly higher gradient density contains a hydrophobic insert (eGFP) (Table 2.8). This result can be explained in terms of the structural nature of the eGFP insert as it forms a highly stable, tight barrel-like structure (Ormo *et al.*, 1996; Wachter *et al.*, 1997; Yang *et al.*, 1996) that could increase the overall density of the tubules, hence increasing the overall density of the chimeric protein. This can also explain why the NS1/eGFP (Figure 2.17) tubules that carry the larger sized insert have a smaller observed diameter (29 nm) than the NS1/VP2-40 and -150 tubules (diameter = 33 nm). The hydrophobic eGFP insert is more densely packaged than

the hydrophilic VP2 inserts, hence having less of an effect on the physical size of the protein it is inserted into.

In general the results from sucrose density gradient studies seem to indicate that the recombinant proteins form particulate structures of some sort, with varying densities and sizes that are related to assembly. If formation of tubules were totally abrogated due to insertion, the proteins would have either concentrated in the top fractions in monomer or dimer forms or aggregated in a dense mass of proteins that would sediment in the pellet fraction. The differences in density and size caused by the physico-chemical properties of the inserts are responsible for the differences in the sucrose gradient distribution profiles of the different NS1 constructs.

Although TEM studies and sucrose density analysis confirm the stability of the tubular presentation system, no conclusions can be drawn about structural and functional integrity of the inserted peptide and proteins. The eGFP protein insert not only served as a reporter protein for monitoring gene expression but fluorescence of the recombinant NS1-eGFP protein also gave an indication of the stability of peptides inserted into the C-terminus region of the NS1 protein. High levels of green fluorescence were observed for the cells expressing the NS1/eGFP protein (Figure 2.18). From this result we can establish that peptides inserted in the NS1 protein remain functionally active even after insertion and that these peptides.

The distribution of NS1/eGFP fluorescence and NS1/eGFP protein concentration in a sucrose density gradient was compared to establish the correlation of fluorescence and NS1/eGFP protein (Figure 2.19). The results from this study confirm that fluorescence correlates with the presence of NS1/eGFP and that the inserted eGFP retains full functionality in the NS1 carrier protein. The fact that levels of fluorescence and protein correlates in the bottom third of the sucrose gradient shows that the inserted eGFP protein is presented on the surface of the tubular structures and is not masked in some way by the assembly of the protein into the tubular structure. In short this study confirms functional integrity of peptides inserted into NS1 as well as presentation of the insert on the surface of the NS1 structure.

Storage stability studies were used to evaluate the stability of wild type and chimeric NS1 proteins after exposure to different temperature conditions that mimic the possible conditions to which a commercial vaccine preparation will be exposed during storage. It is important that the vaccine-associated proteins do not degrade

under the conditions that are being tested. If NS1-derived proteins are not stable under these different conditions, then the NS1 presentation system is not commercially viable, irrespective of immunogenicity.

Overall when looking at the results from storage stability studies, the NS1/VP2-40 and NS1/VP2-150 proteins are most affected by the different storage conditions and sediment in the in fractions at the top of the gradient (Figures 2.21 and 2.22). These results seem to indicate that the three different storage conditions cause longer tubules to depolymerise into smaller tubules and tubule subunits. It seems that the insertion of these specific proteins decreases tubular structural stability as the wild type NS1 protein only shifts marginally upwards in the gradients after treatment, but presumably still form tubules (Figure 2.20). Compared to NS1/VP2-40 and -150, the NS1/eGFP protein is remarkable stable under most storage conditions and like the wild type NS1 protein sediment at a slightly higher position in the gradient. It is only after flash freezing that the majority of the NS1/eGFP protein pellets and probably forms a mass of high density aggregated proteins. The stability of the NS1/eGFP protein can most probably be attributed to the stability of the eGFP insert that probably stabilizes the NS1 protein tubule structure. This insert may not give an accurate indication of the effect of similarly sized insert of other origin on the stability of the NS1 protein due to the inherent structural stability of the eGFP barrel-like structure.

The difference in stability of the three recombinant NS1 proteins under different storage conditions obviates the need to evaluate the storage stability of each construct individually. The method of long term storage of NS1-based protein, if used as vaccines, will be unique for each protein construct. The fact that most of the NS1/VP2-40 and -150 proteins exist as smaller units (shorter tubules and tubule subunits) after storage under these different conditions are not negative as the inserted peptide will be presented in a predictable and structured form, even in tubule subunit form. This might even be beneficial for the elicitation of a humoral response as smaller NS1 protein units can increase antibody binding to the presented tubules. Based on these results it seems as if the recombinant NS1 proteins do not degrade under the different storage conditions. This said, the effect of depolymerisation of proteins to smaller NS1 subunits on the immunogenicity of these proteins should be investigated and compared to that of untreated sample that occur in a predominantly tubular state.

Although similar storage stability studies do not exist for AHSV or BTV NS1, biophysical studies have been performed for both AHSV and BTV NS1 tubules to determine the effects of chemical and physical treatments on tubule morphology (Maree, 2000; Maree, Huisman, 1997; Marshall *et al.*, 1990). In general, these studies suggested that the AHSV tubules are more fragile than BTV tubules. Studies on BTV and AHSV reported that conditions that caused reduction in tubule length were associated with a large increase in the number of circular forms when observed under a transmission electron microscope. These circular forms could represent cross-sections of tubules or very short subsections of larger tubules and have been reported to be an intermediate between BTV NS1 monomers and tubules and were shown to be able to recondense into tubules (Marshall *et al.*, 1990). It is possible that the proteins observed in the top part of gradients following some of the storage stability studies are present in these smaller circular forms. This will be investigated as part of a future study. It is unlikely that the NS1 and NS1 fusion proteins observed in the top fractions of the gradients are in monomeric form as biophysical studies on BTV NS1 tubules showed that it was not possible to recover monomeric NS1 proteins from tubules except under strongly denaturing conditions (e.g. SDS treatment) (Marshall *et al.*, 1990).

Primary phase testing of the NS1 protein showed that the particulate structure remains stable even after insertion of inserts up to 240 amino acids in the internal C-terminal region. The relatively large size of inserts without total abrogation of structure opens the possibility for further studies into the use of the AHSV NS1 protein for the immunogenic presentation of foreign epitopes on the surface of the tubular structures.

In second phase testing of the NS1 protein as a vaccine delivery system the antigenicity and immunogenicity of the chimeric NS1 proteins and inserts were evaluated using Western blot analysis. In order to investigate the immunogenicity of peptides inserted into the C-terminal region of the NS1 protein, guinea pigs were injected with baculovirus-expressed AHSV6 NS1, NS1/VP2-40, -150 and NS1/eGFP (as described in section 2.2.15). Western blotting results showed that the serum generated to the native NS1 was not reactive with NS1 or any of the other constructs, indicating that the NS1 protein might not be highly immunogenic (results not shown). Antibodies in anti-NS1/VP-40 serum reacted not only with the NS1 protein and NS1/VP2-40, but also showed high levels of reaction with NS1/VP2-150 and NS1/eGFP (Figure 2.24). The serum also contained antibodies that reacted with the

VP2 insert presented in the AHSV VP7 protein presentation system (Riley, 2003). The NS1/VP2-150 protein elicited only low levels of antibody response against wt NS1 protein, but not against NS1/VP2-150, the other chimeric NS1 constructs or the inserts presented on the surface of VP7 (Figure 2.25). It is possible that the large size and /or physico-chemical properties of the insert distorts the NS1/VP2-150 structure in such a way that the peptide insert is masked and not accessible to the immune system.

The NS1/eGFP protein elicited antibodies that were able to bind to the eGFP protein, NS1/eGFP and the eGFP protein presented in the AHSV VP7 presentations system (Mizrachi) (Figure 2.26). This confirms the immunogenicity of the eGFP protein presented on the NS1 vector. The fact that antibodies generated by NS1/eGFP are able to react with eGFP, but not NS1 once again confirms that the generated antibodies are directed against to the insert and not the NS1 presentation system. This is supported by the inability of serum generated against NS1 to react with the native NS1 protein (results not shown).

In summary, it appears that the humoral immune response against NS1/VP2-40 and NS1/eGFP are mainly to the inserts and only low levels of antibodies are elicited to the NS1 carrier protein. It also seems that the lack of immune response elicited by the NS1/VP2-150 protein can be due to structural changes caused by the insert. All these conclusions are confirmed by the results obtained from Western blots performed using a polyvalent serum against AHSV serotype 3 probed to native NS1 and the chimeric NS1 constructs. The polyvalent serum against AHSV serotype 3 recognized only wild type NS1 and NS/VP2-40 (Figure 2.27). As the NS1 protein is highly conserved amongst AHSV serotypes and the VP2 protein is highly variable and serotype specific, it is unlikely that the AHSV3 specific serum recognizes the AHSV9 VP2 insert. The failure of the serum to recognize NS1/VP2 -150 and NS1/eGFP suggest that the peptide insert have altered the structure of the carrier NS1 protein in such way that it is no longer recognized by NS1 specific antibodies. These results are in part supported by transmission electron microscopic studies that show altered internal structures for both NS1/VP2-150 and NS1/eGFP.

To determine if the peptides inserted into the NS1 proteins retain their antigenicity, Western blots analysis were performed using serum generated to the native GFP protein (Sigma-Aldrich) and GFP presented in the AHSV VP7 proteins (Mizrachi, unpublished) probed to the chimeric NS1/eGFP protein. Both these sera contained

antibodies that reacted with the eGFP protein inserted into the NS1 protein. This shows that the peptide retains its antigenicity even when inserted into the NS1 protein. No conclusions can be drawn from these results in terms of the structural integrity of the inserts as proteins for Western blotting were resolved using denaturing SDS-PAGE (section 2.2.16).

No further immunological studies were included in this specific investigation, as the primary objectives of this study was the evaluation of the structural stability of NS1 as a peptide presentation system. More detailed immunological studies will form part of secondary phase testing of the NS1 protein. Based on the limited available immunological results it seems as if peptides presented on the surface of the NS1 proteins can elicit an immune response in the form of antibodies that are able to recognize the native peptide. More in depth studies such as ELISA (enzyme-linked immunosorbent assay) and virus protection experiments are necessary before the NS1 protein can be compared to the results from available BTV NS1 protein immunological studies.

These first phase studies have shown that that the AHSV NS1 protein is able to accommodate inserts of between 40 and 240 amino acids and still form tubules spontaneously in recombinant baculovirus-infected Sf9 cells. More importantly these inserts retain functionality and on an immunological level retain their antigenicity. Preliminary immunological studies also indicate that these inserts are immunogenic when presented in the NS1 protein. Based on these results it seems feasible to continue with further development of the AHSV NS1 protein as a carrier molecule for the delivery of foreign epitopes as immunogens.

## CHAPTER 3

### CONCLUDING REMARKS AND FUTURE PROSPECTS

One of the great impediments to the peptide-based subunit vaccine approach is the poor immunogenicity of peptides. This can potentially be overcome by presenting a peptide in the context of a peptide presentation system. A second problem facing peptide vaccines is the elasticity of most pathogens, where quasi-species or mutants can rapidly develop in order to circumvent the immune response directed at a single epitope. Finally, due to the outbred nature of human and animal populations, the specificity of peptide binding to a specific MHC molecule would preclude mass immunization. These last two problems can in part be overcome by the development of “string-of-beads” vaccines containing multiple epitopes (Whitton *et al.*, 1993). A combined solution to these problems would be a protein based peptide presentation system that can augment the immunogenicity of the peptides by presenting them not only on the surface of larger structure, but also in a multi-array on the surface of a multimeric structure such as AHSV NS1 tubules.

The non-structural protein NS1 forms characteristic tubules in the cytoplasm of orbivirus infected cells (Huisman, Els, 1979; Murphy *et al.*, 1971; Thomas, Miller, 1971; Tsai, Karstad, 1970; Tsai, Karstad, 1973b; Verwoerd *et al.*, 1979). The use of NS1 that spontaneously assembles into tubules as a carrier protein is associated with many advantages inherent to the protein such as the high levels of expression and the relative ease of purification). Mikhailov *et al.* (1996) also demonstrated that co-infection of Sf cells with the three different recombinant baculoviruses lead to the formation of chimeric NS1 tubules that contained the corresponding three epitopes (Mikhailov *et al.*, 1996). This observation opens the way for using recombinant NS1 tubules as carriers for the delivery of multiple epitopes and could solve the associated problem of pathogen elasticity and vaccine variability.

In addition to these factors, studies on BTV NS1 have shown that chimeric BTV tubules are able to induce protective cytotoxic T lymphocytes responses to epitopes inserted at the C-terminus of the protein (Ghosh *et al.*, 2002a; Ghosh *et al.*, 2002b; Ghosh *et al.*, 2002c; Larke *et al.*, 2005). As similar studies do not exist for AHSV NS1, this study was initiated to evaluate the potential of NS1 tubules as a multimeric carrier for the delivery of foreign epitopes as immunogens. This study was designed to evaluate the system at two different levels. The first level of testing involved evaluation of structural stability of the NS1 protein. Foreign peptides with sizes corresponding to 40 aa, 150 aa and 250 aa were inserted into an internal NS1 site to



determine if the protein is still able to assemble into a predictable multimeric structure. The second level testing involved evaluating the antigenicity and immunogenicity of foreign peptides presented in the NS1 system. This phase of testing evaluated if the multimeric NS1 structures are able to present foreign antigenic peptides to the immune system on the surface of the NS1 vector.

Based on the above, the investigation of the potential of AHSV NS1 as a peptide vaccine presentation system was launched. This study focused specifically on identifying an appropriate site for peptide insertion in the NS1 protein and evaluating and determining the structural stability of the NS1 protein and associate particulate structures after peptide insertion. Structural analysis of chimeric AHSV6 NS1 proteins with peptide insert of 40, 120 and 240 amino acids into a predicted surface displayed (hydrophobic) C-terminal region, showed that the chimeric proteins retained their ability to assemble spontaneously into tubules with morphologies similar to that of the native NS1 protein. The recombinant proteins spontaneously assembled into distinct tubules with a slightly increased diameter, but the NS1/VP2-150 and NS1/eGFP proteins do not possess the typical internal lumen as observed for AHSV NS1 tubules. Not only are these chimeric NS1 proteins structurally stable, but they are synthesized in large quantities in insect cells infected with recombinant viruses and are easily purified using sucrose gradients. The foreign peptide inserts did not dramatically affect the distribution of particulate structures in sucrose gradients and these sucrose density gradient results indicate that the recombinant NS1 proteins form particles with densities and sizes very similar to that of the wild type NS1 protein and that the ability of these proteins to form tubular structures is not abolished by the insertion of insert of up 240 amino acids.

Preliminary immunological studies indicated that, with the exception of the NS1/VP2-150 protein, the 40 amino acid region of VP2 and the eGFP protein are immunogenic when presented in the NS1 carrier molecule. These two foreign inserts also retained their antigenicity within the NS1 context and was recognized by antibodies targeted specifically to these peptides. The immunogenicity of foreign peptides presented on the NS1 carrier system needs to be tested more accurately using more sensitive methods. Firstly, the antisera raised against the chimeric NS1 proteins need to be tested using ELISA incorporating AHSV9 VP2 antigen and eGFP protein, respectively, to determine the titers of these antisera. For the antiserum obtained against NS1/VP2-40, a virus neutralization assay would give a more conclusive result to whether the neutralising antibodies were raised against AHSV serotype 9.

Future studies should focus on the use of inserts that are more immunologically defined and has proven immune response such as the virus neutralising region identified for AHSV9 VP2 (Martinez-Torrecuadrada, Casal, 1995); the FMDV VP1 peptide (aa135-144).

This study also focused on only one insertion site in the NS1 protein that is located internally in the C-terminal region of the protein. Studies on BTV NS1 showed that foreign sequences attached at the C-terminus of the protein were able to elicit both cellular and humoral response (Ghosh *et al.*, 2002a; Ghosh *et al.*, 2002b; Ghosh *et al.*, 2002c; Larke *et al.*, 2005). Studies are already in progress that will investigate and compare the capabilities of the different cloning sites in the NS1 protein in terms of structural stability and insert presentation.

Another aspect of the NS1 presentation system that requires further investigation is the effect of storage conditions on the immunogenicity of the chimeric NS1 proteins. Results from storage stability studies indicate that the storage conditions cause the chimeric NS1 proteins to depolymerize into smaller units possibly tubule subunits and NS1 dimers. It is of paramount importance that the immunogenicity of these proteins is compared before and after storage at the tested conditions. If storage renders these chimeric proteins unable to elicit an immune response, they are of limited commercial value as a vaccine presentation system. This is unlikely as immunological studies on the AHSV VP7 presentation system have indicated that smaller subunits of the protein that usually form multimeric crystal, elicit a much higher immune response (Dr. W. Fick, personal communication).

Future studies will also include co-infection of Sf9 cells with different recombinant baculoviruses that express chimeric NS1 tubules carrying different epitopes that could lead to the formation of chimeric NS1 tubules that display the corresponding epitopes (Mikhailov *et al.*, 1996). If this approach is successful it has to potential to be used as a vaccine system for multi-serotype viruses such as AHSV and simultaneous vaccination against multiple pathogens.

In conclusion, the AHSV NS1 protein is structurally stable even after insertion of peptides up to 240 amino acids into a hydrophilic domain in the C-terminal region of the protein. These chimeric proteins are still able form recognizable tubules with morphology similar to that of AHSV6 NS1. The foreign peptides inserted into this

internal site are presented on the NS1 protein in such a way that they retain structural and functional integrity and have been shown to be both antigenic and immunogenic. These results justify the further instigation of the AHSV NS1 protein as carrier protein for the immunogenic display of foreign peptides.

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## Appendices

### Appendix A-1. CLUSTAL X (1.8) Nucleotide sequence

alignment of the sequenced NS1 gene with AHSV6 NS1, AHSV9 NS1 and AHSV4 NS1

```
seq
ATGGATAGGTTCTTGACTTATTTCCAGGTACGAGGAGAGAGAGCAAACGCTGTTCCGGCTG 60
AHSV6
ATGGATAGGTTCTTGACTTATTTCCAGGTACGAGGAGAGAGAGCAAACGCTGTTCCGGCTG 60
AHSV4
ATGGATAAGTTCTTGACTTATTTCCAGGTACGAGGAGAGAGAGCAAACGCTGTTCCGGCTG 60
AHSV9
ATGGATAGGCTCTTGACTTATTTCCAGGTACGAGGGGAGAGAGCAAACGCTGTTCCGGCTG 60
***** * *****
*****
```

```
seq
TTTGGAGAGATTTCCGAACAAATAGATTGCTCACATCTCAAACGAGATTGCTTTGTAAAT 120
AHSV6
TTTGGAGAGATTTCCGAACAAATAGATTGCTCACATCTCAAACGAGATTGCTTTGTAAAT 120
AHSV4
TTTGGAGAGATTTCCGAACAAATAGATTGCTCACATCTCAAACGAGATTGCTTTGTAAAT 120
AHSV9
TTTGGAGAGATTTCCGAACAAATAGATTGCTCACATCTCAAACGAGATTGCTTTGTAAAT 120
*****
```

```
seq
GGAATATGTGCAAGACAACACTTTAAAGAATGCTGTAATATTGCTACAGATAATGGCTCA 180
AHSV6
GGAATATGTGCAAGACAACACTTTAAAGAATGCTGTAATATTGCTACAGATAATGGCTCA 180
AHSV4
GGAATATGTGCAAGACAACACTTTAAAGAATGCTGTAATATTGCTACAGATAATGGCTCA 180
AHSV9
GGAATATGTGCAAGACAACACTTTAAAGAATGCTGTAATATTGCTACAGATAATGGCTCA 180
*****
```

```
seq
CGCACAAATGCAGATAAACTAGTGGCTTTAGCTTTGCGAGCACTTTTAGGTAGACAAACT 240
AHSV6
CGCACAAATGCAGATAAACTAGTGGCTTTAGCTTTGCGAGCACTTTTAGATAGACAAACT 240
AHSV4
CGCACAAACGCAGATAAATTAGTGGCTTTAGCTTTGCGAGCACTTTTAGATAGACAAACT 240
AHSV9
CGCACGAACGCAGATAAATTAGTAGCTTTAGCTATGCGAGCGCTTTTAGATAGACAAACT 240
***** ** ***** * * ***** * * *****
*****
```

```
seq
ATTTGGACTTGTGTCATCAAAAATGCGGATTACGTTAGTCAATATGCTGATGAGCAGATG 300
AHSV6
ATTTGGACTTGTGTCATCAAAAATGCGGATTACGTTAGTCAATATGCTGATGAGCAGATG 300
AHSV4
ATTTGGACTTGTGTCATCAAAAATGCGGATTACGTTAGTCAATATGCTGATGAACAGATG 300
```

AHSV9  
ATTTGGGCTTGTGTCATCAAGAATGCGGATTACGTTAGTCAATATGCTGATGAGCAGATG 300  
\*\*\*\*\*

seq  
GAGGAAGAAGTTAATAGGCTGTATGATGTCTATCTCCAGAGCGGGACGAGAGAGGAATTT 360  
AHSV6  
GAGGAAGAAGTTAATAAGCTGTATGATGTCTATCTCCAGAGCGGGACGAGAGAGGAATTT 360  
AHSV4  
GAGGAAGAAGTTAATAAGCTGTATGATGTCTATCTCCAGAGCGGGACGAGAGAGGAATTT 360  
AHSV9  
GAGGAAGAAGTTAATAAACTGTATGATGTCTATCTCCAGAGCGGGACGAGAGAGGAATTT 360  
\*\*\*\*\*

seq  
GAAGGATTTAGACAGAGGAATAGACCGAGTAGAGTTGTGATGGATGATAGCTGCTCAATG 420  
AHSV6  
GAAGGATTTAGACAGAGGAATAGACCGAGTAGAGTTGTGATGGATGATAGCTGCTCAATG 420  
AHSV4  
GAAGGATTTAGACAGAGGAATAGACCAAGTAGAGTTGTGATGGATGATAGCTGCTCAATG 420  
AHSV9  
GAAGGATTTAGACAGAGAAGTAGACCAAGTAGAGTCGTGATGGATGATAGTTGTTCAATG 420  
\*\*\*\*\*

seq  
CTCTCATATTTTTACATTCCAATGAATCAAGGGAATCCAGCTCCAGTTGCGAAGCTTAGC 480  
AHSV6  
CTCTCATATTTTTACATTCCAATGAATCAAGGGAATCCAGCTCCAGTTGCGAAGCTTAGC 480  
AHSV4  
CTCTCATATTTTTACATTCCAATGAATCAAGGGAATCCAGCTCCAGTTGCGAAGCTTAGC 480  
AHSV9  
CTCTCATATTTTTACATTCCAATGAATCAAGGAAATCCAGCTCCAGTTGCAAAGCTTAGC 480  
\*\*\*\*\*

seq  
CGATGGGGTCAATTTGGAATTTGTTACTATGATAGAACAAAATGTTGATGGATTGATTCCG 540  
AHSV6  
CGATGGGGTCAATTTGGAATTTGTTACTATGATAGAACAAAATGTTGATGGATTGATTCCG 540  
AHSV4  
CGATGGGGTCAATTTGGAATTTGTTACTATGACAGAACAAAATGTTGATGGATTGATTCCG 540  
AHSV9  
CGATGGGGTCAATTTGGAATTTGTTACTATGATAGACAAAATGTTGATGGATTGATTCCA 540  
\*\*\*\*\*

seq  
TATGATGAGATCGGTTTGTAGCTCAAGCTATAGACGGCCTAAAGGATCTGATTGAAGGGCGA 600  
AHSV6  
TATGATGAGATCGGTTTGTAGCTCAAGCTATAGACGGCCTAAAGGATCTGATTGAAGGGCGA 600  
AHSV4  
TATGATGAGATCGGTTTGTAGCTCAAGCTATAGATGGTTTAAAGGATCTGATCGAAGGGCGA 600  
AHSV9  
TATGATGAAATCGGTTTGTAGCTCAAGCTATAGATGGTCTAAAAGATTTGATTGAAGGGCGA 600  
\*\*\*\*\*

```
seq
TTGCCCGTTTGCCCTTATACTGGAGCGAATGGTAGAATTAATGCTGTTTTACATTTACCA 660
AHSV6
TTGCCCGTTTGCCCTTATACTGGAGCGAATGGTAGAATTAATGCTGTTTTACATTTACCA 660
AHSV4
TTGCCCGTTTGCCCTTATACTGGAGCGAATGGCAGGATTAATGCTGTTTTACATCTACCA 660
AHSV9
TTGCCTGTTTGCCCTTATACTGGAGTGAATAGCAGGATTAATGCTGTTTTACATCTACCG 660
*****
*****
```

```
seq
TTAGAGATGGAGGTGATTATGGCGGTGCAGGAAAATGCAACACAATTAATGCGTAGAGCG 720
AHSV6
TTAGAGATGGAGGTGATTATGGCGGTGCAGGAAAATGCAACACAATTAATGCGTAGAGCG 720
AHSV4
TTAGAGATGGAAGTGATTATGGCGGTGCAGGAAAATGCGACACAATTAATGCGTAGAGCG 720
AHSV9
TTAGAAATGGAAGTGATAATGGCAGTGCAGGAAAATGCTACACAATTAATGCGCAGAGCA 720
*****
*****
```

```
seq
GCACAGGATTTCAAATTCATCACACATGCTGGATGGAGGCTATATCCAAGATTGTTGCGA 780
AHSV6
GCACAGGATTTCAAATTCATCACACATGCTGGATGGAGGCTATATCCAAGATTGTTGCGA 780
AHSV4
GCACAGGATTTCAAATTCATTACACATGCTGGATGGAAGTTATACCCAAGATTGTTGCGA 780
AHSV9
GCACAGGATTTCAAATTCATCACACACGCTGGATGGAAGCTATACCCAAGATTGTTGCGA 780
*****
*****
```

```
seq
CAACGGTTCGCGATCGAGGACGCTACGGAGGGGGTGATTCATCATGTGATGCTAGGCCAT 840
AHSV6
CAACGGTTCGCGATCGAGGACGCTACGGAGGGGGTGATTCATCATGTGATGCTAGGCCAT 840
AHSV4
CAACGGTTCGCGATCGAGGACGCAACGGAAGGGTGATTCATCATGTGATGCTAGGCCAT 840
AHSV9
CAACGTTTTGCGATCGGAGACGCTTTCGGAGGGGGTGATTCATCATGTGATGCTTGGCCAT 840
*****
*****
```

```
seq
TTAAGATATTATGATGAAGATAACAAGTATCGTGAAGTATCGCTTCCTTAACGATGGATCT 900
AHSV6
TTAAGATATTATGATGAAGATAACAAGTATCGTGAAGTATCGCTTCCTTAACGATGGATCT 900
AHSV4
TTAAGATATTATGATGAAGATAACAAGTATCGTGAAGTATCGCTTCCTTAACGATGGATCT 900
AHSV9
TTAAGATATTATGATGGAGATACGAGTATCGTAAAGTATTGCTTCCTTTACGATGGATCT 900
*****
*****
```

```
seq
TTAGATTGGAGGACTTGGACAATTCCTTTACATCTGATGCGGACAGCAAGGTTGGGACAT 960
AHSV6
TTAGATTGGAGGACTTGGACAATTCCTTTACATCTGATGCGGACAGCAAGGTTGGGACAT 960
AHSV4
TTAGATTGGAGGACTTGGACAATTCCTTTACATCTGATGCGGACAGCAAGGTTGGGACAT 960
```

```

AHSV9
CTAGATTGGAGGACTTGGACAGTTCCCTTTACACCTGATGAGGACAGCGAGGTTGGGACAT 960
*****
*****

seq
CTGCAACCGGAATCAATTTTAGTCTTTATGCATAAAAAGCTCACATGTCAGGTATGCTTT 1020
AHSV6          CTGCAACCGGAATCAATTTTAGTCTTTATGCATAAAA-
GCCTACATGTCAGGTATGCTTT 1019
AHSV4
CTGCAACCGGAATCAATTTTAGTCTTTATGCATAAAAAGCTCACATGTCAGGTATGTTTT 1020
AHSV9          CTGCAACCGGAATCAATTTTGTCTTTATGCATAAAA-
GCTTACGTGTCAGGTATGTTTT 1019
*****
*****

seq
ATGGTTGACCTCGCTCTGCTTGACACAATCCCGGTGGTTGATTCAAAGATTGC-TGAACT 1079
AHSV6
ATGGTTGACCTCGCTCTGCTTGACACAATCCCGGTGGTTGATTCAAAGTTGCCTGAACT 1079
AHSV4
ATGGTTGACCTCGCTATGCTTGACACAATCCAGTGGTTGATTCAAAGTTGC-TGAACT 1079
AHSV9
ATGGTTGACCTCGCTCTGCTTGACACAATCCAGTGGTTGATTCAAAAATTGCCTGAACT 1079
*****
*****

seq
AACAGGAGGCACAGATGTATTTTATACACGTGCGTATGTACATGCGGACAATCACAAAGT 1139
AHSV6
AACAGGAGGCACAGATGTACTTTTATACACGTGCGTATGTACATGCGGACAATCACAAAGT 1139
AHSV4
AACAGGAGGCACAGATGTATTTTATACACGTGCGTATGTACATGCGGACAATCACAAAGT 1139
AHSV9
AACAGGGGGCACAGATGTGTTTCTATACACGCGGTATGTACATGCGGAGAATCACAAAGT 1139
*****
*****

seq
GCCAAATGTCAGAGATTTGATGATGAATGAAGTCTTCAGGAAGATCGATGATCATTGGGT 1199
AHSV6
GCCAAATGTCAGAGATTTGATGATGAATGAAGTCTTCAGGAAGATCGATGATCATTGGGT 1199
AHSV4
GCCAAATGTCAGAGATTTGATGATGAATGAAGTCTTCAGGAAGATCGATGATCATTGGGT 1199
AHSV9
GCCAAATGACAGAGATTTGATGATGAATGAAGTCTTCAGGAAGATCGATGATCATTGGGT 1199
*****
*****

seq
GATTCAGAAGTGTACATAACGAAGGAAGCGATTACTGTAAGTCAATTCAGATTCAGAG 1259
AHSV6
GATTCAGAAGTGTACATAACGAAGGAAGCGATTACTGTAAGTCAATTCAGATTCAGAG 1259
AHSV4
GATTCAGAAGTGTACATAACGAAGGAAGCGATTACTGTAAGTCAATTCAGATCCAGAG 1259
AHSV9
GATCCAGAAGTGTACATAACGAAGGAAGCGATTACTGTAACCGCAATTCAGATCCAGAG 1259
*****
*****

```

seq  
GTCGATCAGAGGTGATGGGCAGTGGGATACTCCGATGTTTCACCAATCAATGGCTCTGTT 1319  
AHSV6  
GTCGATCAGAGGTGATGGGCAGTGGGATACTCCGATGTTTCACCAATCAATGGCTCTGTT 1319  
AHSV4  
GTCGATCAGAGGTGATGGGCAGTGGGATACTCCGATGTTTCACCAATCAATGGCTCTGTT 1319  
AHSV9  
GTCAATCAGAGGTGATGGGCAGTGGGATACTCCGATGTTTCACCAATCAATGGCTCTGTT 1319

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seq  
AACACGATTGATTGTTTATTGGTTAACGGATGTGACTGAGAGAAGTGCTATCTTTCGGCT 1379  
AHSV6  
AACACGATTGATTGTTTATTGGTTAACGGATGTGACTGAGAGAAGTGCTATCTTTCGGCT 1379  
AHSV4  
AACACGATTGATTGTTTATTGGTTAACGGATGTGACAGAGAGAAGCGCTATCTTTCGGCT 1379  
AHSV9  
AACACGATTGATTGTTTATTGGTTAACAGATGTGACTGAGAGAAGCGCTATCTTTCGGCT 1379

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\*\*\*\*\*

seq  
GACTTGTTTCGCAATCTTTCGGATGTAAGCCAACGGCTCGAGGTAGATATATTGATTGGGA 1439  
AHSV6  
GACTTGTTTCGCAATCTTTCGGATGTAAGCCAACGGCTCGAGGTAGATATATTGATTGGGA 1439  
AHSV4  
GACTTGTTTCGCAATCTTTCGGATGTAAGCCAACAGCTCGAGGTAGATATATTGATTGGGA 1439  
AHSV9  
GACTTGTTATGCAATCTTTCGGATGTAAGCCAACAGCTCGAGGTAGATATATTGATTGGGA 1439

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seq  
TGATCTTGGAACATTCATGAAGAATGTCTTGGATGGAAGAGATTTGACTGTTTTGGAAGA 1499  
AHSV6  
TGATCTTGGAACATTCATGAAGAATGTCTTGGATGGAAGAGATTTGACTGTTTTGGAAGA 1499  
AHSV4  
TGATCTTGGAACATTCCTTGAAGAATGTGTTGGATGGAAGAGATTTGACTGTTTTGGAAGA 1499  
AHSV9  
CGATCTTGGAACGTTTCATGAAAAATGTGTTAGATGGAAGAGACTTGACTGTTTTGGAAGA 1499

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seq  
TGAGACATGTTTTATTTTCGATGATGAGGATGGCGATGTTGCATGTGCAGAGATCCAAGGT 1559  
AHSV6  
TGAGACATGTTTTATTTTCGATGATGAGGATGGCGATGTTGCATGTGCAGAGATCCAAGGT 1559  
AHSV4  
TGAAACATGTTTTATTTTCGATGATGAGGATGGCGATGTTGCATGTGCAGAGATCCAAGGC 1559  
AHSV9  
TGAGACATGTTTTATTTTCGATGATGAGGATGGCGATGTTGCATGTGCGGAGATCCAAGGT 1559

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\*\*\*\*\*

seq  
AGTGTGCGCAACTGTGTTGGAGGCGCCATTAGAAAATACAACAGGTTGGCCAGATCGTTGA 1619  
AHSV6  
AGTGTGCGCAACTGTGTTGGAGGCGCCATTAGAAAATACAACAGGTTGGCCAGATCGTTGA 1619  
AHSV4  
AGTGTGCGCAACTGTGTTGGAGGCGCCATTGAAAATACAACAGGTTGGCCAGATCGTTGA 1619

AHSV9  
AGTGTGCGCAACTGTGTTGGAGGCGCCATTGGAAATACAACAGGTTGGTCAGATCGTTGA 1619  
\*\*\*\*\*

seq AGTACCCTTTGATTTTATGCATAATTAG 1647  
AHSV6 AGTACCCTTTGATTTTATGCATAATTAG 1647  
AHSV4 AGTACCCTTTGATTTTATGCATAATTAG 1647  
AHSV9 GGTACCCTTTGATTTTATGCATAATTAG 1647  
\*\*\*\*\*

**Appendix A-2.** CLUSTAL X (1.8) Multiple nucleotide sequence alignment of AHSV6 NS1 with NS1/VP2-40 and NS1/VP2-150.

```

NS1/VP2-40
ATGGATAGGTTCTTGACTTATTTCCAGGTACGAGGAGAGAGAGCAAACGCTGTTCCGGCTG 60
NS1/VP2-150
ATGGATAGGTTCTTGACTTATTTCCAGGTACGAGGAGAGAGAGCAAACGCTGTTCCGGCTG 60
AHSV6
ATGGATAGGTTCTTGACTTATTTCCAGGTACGAGGAGAGAGAGCAAACGCTGTTCCGGCTG 60

```

\*\*\*\*\*

```

NS1/VP2-40
TTTGGAGAGATTTCCGAACAAATAGATTGCTCACATCTCAAACGAGATTGCTTTGTAAAT 120
NS1/VP2-150
TTTGGAGAGATTTCCGAACAAATAGATTGCTCACATCTCAAACGAGATTGCTTTGTAAAT 120
AHSV6
TTTGGAGAGATTTCCGAACAAATAGATTGCTCACATCTCAAACGAGATTGCTTTGTAAAT 120

```

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```

NS1/VP2-40
GGAATATGTGCAAGACAACACTTTAAAGAATGCTGTAATATTGCTACAGATAATGGCTCA 180
NS1/VP2-150
GGAATATGTGCAAGACAACACTTTAAAGAATGCTGTAATATTGCTACAGATAATGGCTCA 180
AHSV6
GGAATATGTGCAAGACAACACTTTAAAGAATGCTGTAATATTGCTACAGATAATGGCTCA 180

```

\*\*\*\*\*

```

NS1/VP2-40
CGCACAAATGCAGATAAACTAGTGGCTTTAGCTTTGCGAGCACTTTTAGGTAGACAAACT 240
NS1/VP2-150
CGCACAAATGCAGATAAACTAGTGGCTTTAGCTTTGCGAGCACTTTTAGGTAGACAAACT 240
AHSV6
CGCACAAATGCAGATAAACTAGTGGCTTTAGCTTTGCGAGCACTTTTAGGTAGACAAACT 240

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\*\*\*\*\*

```

NS1/VP2-40
ATTTGGACTTGTGTTCATCAAAAATGCGGATTACGTTAGTCAATATGCTGATGAGCAGATG 300
NS1/VP2-150
ATTTGGACTTGTGTTCATCAAAAATGCGGATTACGTTAGTCAATATGCTGATGAGCAGATG 300
AHSV6
ATTTGGACTTGTGTTCATCAAAAATGCGGATTACGTTAGTCAATATGCTGATGAGCAGATG 300

```

\*\*\*\*\*

```

NS1/VP2-40
GAGGAAGAAGTTAATAGGCTGTATGATGTCTATCTCCAGAGCGGGACGAGAGAGGAATTT 360
NS1/VP2-150
GAGGAAGAAGTTAATAGGCTGTATGATGTCTATCTCCAGAGCGGGACGAGAGAGGAATTT 360
AHSV6
GAGGAAGAAGTTAATAGGCTGTATGATGTCTATCTCCAGAGCGGGACGAGAGAGGAATTT 360

```

\*\*\*\*\*



NS1/VP2-40  
GAAGGATTTAGACAGAGGAATAGACCGAGTAGAGTTGTGATGGATGATAGCTGCTCAATG 420  
NS1/VP2-150  
GAAGGATTTAGACAGAGGAATAGACCGAGTAGAGTTGTGATGGATGATAGCTGCTCAATG 420  
AHSV6  
GAAGGATTTAGACAGAGGAATAGACCGAGTAGAGTTGTGATGGATGATAGCTGCTCAATG 420

\*\*\*\*\*

NS1/VP2-40  
CTCTCATATTTTTACATTCCAATGAATCAAGGGAATCCAGCTCCAGTTGCGAAGCTTAGC 480  
NS1/VP2-150  
CTCTCATATTTTTACATTCCAATGAATCAAGGGAATCCAGCTCCAGTTGCGAAGCTTAGC 480  
AHSV6  
CTCTCATATTTTTACATTCCAATGAATCAAGGGAATCCAGCTCCAGTTGCGAAGCTTAGC 480

\*\*\*\*\*

NS1/VP2-40  
CGATGGGGTCAATTTGGAATTTGTTACTATGATAGAACAAATGTTGATGGATTGATTCCG 540  
NS1/VP2-150  
CGATGGGGTCAATTTGGAATTTGTTACTATGATAGAACAAATGTTGATGGATTGATTCCG 540  
AHSV6  
CGATGGGGTCAATTTGGAATTTGTTACTATGATAGAACAAATGTTGATGGATTGATTCCG 540

\*\*\*\*\*

NS1/VP2-40  
TATGATGAGATCGGTTTAGCTCAAGCTATAGACGGCCTAAAGGATCTGATTGAAGGGCGA 600  
NS1/VP2-150  
TATGATGAGATCGGTTTAGCTCAAGCTATAGACGGCCTAAAGGATCTGATTGAAGGGCGA 600  
AHSV6  
TATGATGAGATCGGTTTAGCTCAAGCTATAGACGGCCTAAAGGATCTGATTGAAGGGCGA 600

\*\*\*\*\*

NS1/VP2-40  
TTGCCCGTTTGCCCTTATACTGGAGCGAATGGTAGAATTAATGCTGTTTTACATTTACCA 660  
NS1/VP2-150  
TTGCCCGTTTGCCCTTATACTGGAGCGAATGGTAGAATTAATGCTGTTTTACATTTACCA 660  
AHSV6  
TTGCCCGTTTGCCCTTATACTGGAGCGAATGGTAGAATTAATGCTGTTTTACATTTACCA 660

\*\*\*\*\*

NS1/VP2-40  
TTAGAGATGGAGGTGATTATGGCGGTGCAGGAAAATGCAACACAATTAATGCGTAGAGCG 720  
NS1/VP2-150  
TTAGAGATGGAGGTGATTATGGCGGTGCAGGAAAATGCAACACAATTAATGCGTAGAGCG 720  
AHSV6  
TTAGAGATGGAGGTGATTATGGCGGTGCAGGAAAATGCAACACAATTAATGCGTAGAGCG 720

\*\*\*\*\*

NS1/VP2-40  
GCACAGGATTTCAAATTCATCACACATGCTGGATGGAGGCTATATCCAAGATTGTTGCGA 780  
NS1/VP2-150  
GCACAGGATTTCAAATTCATCACACATGCTGGATGGAGGCTATATCCAAGATTGTTGCGA 780  
AHSV6  
GCACAGGATTTCAAATTCATCACACATGCTGGATGGAGGCTATATCCAAGATTGTTGCGA 780

\*\*\*\*\*

NS1/VP2-40  
CAACGGTTTCGCGATCGAGGACGCTACGGAGGGGGTGATTCATCATGTGATGCTAGGCCAT 840  
NS1/VP2-150  
CAACGGTTTCGCGATCGAGGACGCTACGGAGGGGGTGATTCATCATGTGATGCTAGGCCAT 840  
AHSV6  
CAACGGTTTCGCGATCGAGGACGCTACGGAGGGGGTGATTCATCATGTGATGCTAGGCCAT 840

\*\*\*\*\*

NS1/VP2-40  
TTAAGATATTATGATGAAGATAACAAGTATCGTGAAGTATCGCTTCCTTAACGATGGATCT 900  
NS1/VP2-150  
TTAAGATATTATGATGAAGATAACAAGTATCGTGAAGTATCGCTTCCTTAACGATGGATCT 900  
AHSV6  
TTAAGATATTATGATGAAGATAACAAGTATCGTGAAGTATCGCTTCCTTAACGATGGATCT 900

\*\*\*\*\*

NS1/VP2-40  
TTAGATTGGAGGACTTGGACAATTCCTTTACATCTGATGCGGACAGCAAGGTTGGGACAT 960  
NS1/VP2-150  
TTAGATTGGAGGACTTGGACAATTCCTTTACATCTGATGCGGACAGCAAGGTTGGGACAT 960  
AHSV6  
TTAGATTGGAGGACTTGGACAATTCCTTTACATCTGATGCGGACAGCAAGGTTGGGACAT 960

\*\*\*\*\*

NS1/VP2-40  
CTGCAACCGGAATCAATTTTAGTCTTTATGCATAAAAAAGCTCACATGTCAGGTATGCTTT 1020  
NS1/VP2-150  
CTGCAACCGGAATCAATTTTAGTCTTTATGCATAAAAAAGCTCACATGTCAGGTATGCTTT 1020  
AHSV6  
CTGCAACCGGAATCAATTTTAGTCTTTATGCATAAAAAAGCTCACATGTCAGGTATGCTTT 1020

\*\*\*\*\*

NS1/VP2-40  
ATGTTGACCTCGCTCTGCTTGACACAATCCCGGTGGTTGATTCAAAGATTGCTGAACTA 1080  
NS1/VP2-150  
ATGTTGACCTCGCTCTGCTTGACACAATCCCGGTGGTTGATTCAAAGATTGCTGAACTA 1080  
AHSV6  
ATGTTGACCTCGCTCTGCTTGACACAATCCCGGTGGTTGATTCAAAGATTGCTGAACTA 1080

\*\*\*\*\*

NS1/VP2-40  
ACAGGAGGCACAGATGTATTTTATACACGTGCGTATGTACATGCGGACAATCACAAAGTG 1140  
NS1/VP2-150  
ACAGGAGGCACAGATGTATTTTATACACGTGCGTATGTACATGCGGACAATCACAAAGTG 1140  
AHSV6  
ACAGGAGGCACAGATGTATTTTATACACGTGCGTATGTACATGCGGACAATCACAAAGTG 1140

\*\*\*\*\*

NS1/VP2-40  
CCAAATGTCAGAGATTTGATGATGAATGAAGTCTTCAGGAAGATCGATGATCATTGGGTG 1200  
NS1/VP2-150  
CCAAATGTCAGAGATTTGATGATGAATGAAGTCTTCAGGAAGATCGATGATCATTGGGTG 1200

AHSV6  
CCAAATGTCAGAGATTTGATGATGAATGAAGTCTTCAGGAAGATCGATGATCATTGGGTG 1200

\*\*\*\*\*

NS1/VP2-40  
ATTCAGAAGTGTTCATACAACGAAGGAAGCGATTACTGTAAGTCAATTCAGATTCAGAGG 1260

NS1/VP2-150  
ATTCAGAAGTGTTCATACAACGAAGGAAGCGATTACTGTAAGTCAATTCAGATTCAGAGG 1260

AHSV6  
ATTCAGAAGTGTTCATACAACGAAGGAAGCGATTACTGTAAGTCAATTCAGATTCAGAGG 1260

\*\*\*\*\*

NS1/VP2-40  
TCGATCAGAGGTGATGGGCAGTGGGATACTCCGATGTTTCACCAATCAATGGCTCTGTTA 1320

NS1/VP2-150  
TCGATCAGAGGTGATGGGCAGTGGGATACTCCGATGTTTCACCAATCAATGGCTCTGTTA 1320

AHSV6  
TCGATCAGAGGTGATGGGCAGTGGGATACTCCGATGTTTCACCAATCAATGGCTCTGTTA 1320

\*\*\*\*\*

NS1/VP2-40  
ACACGATTGATTGTTTATTGGTTAACGGATGTGACTGAGAGAAGTGCTATCTTTCGGCTG 1380

NS1/VP2-150  
ACACGATTGATTGTTTATTGGTTAACGGATGTGACTGAGAGAAGTGCTATCTTTCGGCTG 1380

AHSV6  
ACACGATTGATTGTTTATTGGTTAACGGATGTGACTGAGAGAAGTGCTATCTTTCGGCTG 1380

\*\*\*\*\*

NS1/VP2-40            ACTTGTTTCGCAATCTTCGGATGTAAGCCAACGGCTCGAGCC-----  
----- 1422

NS1/VP2-150  
ACTTGTTTCGCAATCTTCGGATGTAAGCCAACGGCTCGAGCCGAAGACGAAGAATTGAGT 1440

AHSV6                ACTTGTTTCGCAATCTTCGGATGTAAGCCAACGGCTCGAG-----  
----- 1420

\*\*\*\*\*

NS1/VP2-40            -----  
-----

NS1/VP2-150  
CGGAAATAATAGTTGCGGTGATCAATTATGGTTCGAAGTTCGGGACTAGGTCCGGGAAG 1500

AHSV6                -----  
-----

NS1/VP2-40            -----  
-----

NS1/VP2-150  
AAAAAAGATTTGATGACCATTGATAAGCTTGAAAAATACTGTGAATCCTTAACGACGTTT 1560

AHSV6                -----  
-----

NS1/VP2-40            -----  
-----

NS1/VP2-150  
GTTTACAAAAAGAAGCGTGATGAAGGTGATGATGAAACAGCTAGGGCTATCATTAGGAAT 1620

AHSV6 -----  
-----

NS1/VP2-40 -----  
-----

NS1/VP2-150  
CAATGGATTAAGGGAATGCCAAGCATGAATTTGAAAAAAGAAATGAAAGTTTCGCGTGGT 1680  
AHSV6 -----  
-----

NS1/VP2-40 -----  
CTTGAGGTGTTTTAAACGTAATAATAAAAGTT 1452  
NS1/VP2-150  
CCTATTCAAATTTGGTCGTTTTTTTATGTCCCTTGAGGTGTTTTAAACGTAATAATAAAAGTT 1740  
AHSV6 -----  
-----

NS1/VP2-40  
GATATTGATCCAAATCATGATACATGGAAAAACCATGTCAAAGAAATCAGAGAGAGGATG 1512  
NS1/VP2-150  
GATATTGATCCAAATCATGATACATGGAAAAACCATGTCAAAGAAATCAGAGAGAGGATG 1800  
AHSV6 -----  
-----

NS1/VP2-40 CAGAAAGAACAGAGCGCGAATTCCAACCTA-----  
----- 1542  
NS1/VP2-150  
CAGAAAGAACAGAGCGCGAATTCCAACCTTACCCCTTAAAGATCCCAAGTTGATGGCGTCA 1860  
AHSV6 -----  
-----

NS1/VP2-40 -----  
GCTCGAGGTAGATATATTGATTGGGATGATCTTGGAACATTCATG 1587  
NS1/VP2-150  
GCTTATCTACAAGTGGCTCGAGGTAGATATATTGATTGGGATGATCTTGGAACATTCATG 1920  
AHSV6 -----  
GTAGATATATTGATTGGGATGATCTTGGAACATTCATG 1458

\*\*\*\*\*

NS1/VP2-40  
AAGAATGTCTTGATGGAAGAGATTTGACTGTTTTGGAAGATGAGACATGTTTTATTTTCG 1647  
NS1/VP2-150  
AAGAATGTCTTGATGGAAGAGATTTGACTGTTTTGGAAGATGAGACATGTTTTATTTTCG 1980  
AHSV6  
AAGAATGTCTTGATGGAAGAGATTTGACTGTTTTGGAAGATGAGACATGTTTTATTTTCG 1518

\*\*\*\*\*

NS1/VP2-40  
ATGATGAGGATGGCGATGTTGCATGTGCAGAGATCCAAGGTAGTGTGCGCAACTGTGTTG 1707  
NS1/VP2-150  
ATGATGAGGATGGCGATGTTGCATGTGCAGAGATCCAAGGTAGTGTGCGCAACTGTGTTG 2040  
AHSV6  
ATGATGAGGATGGCGATGTTGCATGTGCAGAGATCCAAGGTAGTGTGCGCAACTGTGTTG 1578

\*\*\*\*\*

NS1/VP2-40  
GAGGCGCCATTAGAAATACAACAGGTTGGCCAGATCGTTGAAGTACCCTTTGATTTTATG 1767  
NS1/VP2-150  
GAGGCGCCATTAGAAATACAACAGGTTGGCCAGATCGTTGAAGTACCCTTTGATTTTATG 2100  
AHSV6  
GAGGCGCCATTAGAAATACAACAGGTTGGCCAGATCGTTGAAGTACCCTTTGATTTTATG 1638

\*\*\*\*\*

NS1/VP2-40	CATAATTAG	1776
NS1/VP2-150	CATAATTAG	2109
AHSV6	CATAATTAG	1647
	*****	

**Appendix A-3.** CLUSTAL X (1.8) Multiple nucleotide sequence alignment of NS1/eGFP with eGFP.

NS1/eGFP  
ATGGATAGGTTCTTGACTTATTTCCAGGTACGAGGAGAGAGCAAACGCTGTTCCGGCTG 60  
eGFP -----  
-----

NS1/eGFP  
TTTGGAGAGATTTCCGAACAAATAGATTGCTCACATCTCAAACGAGATTGCTTTGTAAAT 120  
eGFP -----  
-----

NS1/eGFP  
GGAATATGTGCAAGACAACACTTTAAAGAATGCTGTAATATTGCTACAGATAATGGCTCA 180  
eGFP -----  
-----

NS1/eGFP  
CGCACAAATGCAGATAAACTAGTGGCTTTAGCTTTGCGAGCACTTTTAGGTAGACAAACT 240  
eGFP -----  
-----

NS1/eGFP  
ATTTGGACTTGTGTCATCAAAAATGCGGATTACGTTAGTCAATATGCTGATGAGCAGATG 300  
eGFP -----  
-----

NS1/eGFP  
GAGGAAGAAGTTAATAGGCTGTATGATGTCTATCTCCAGAGCGGGACGAGAGAGGAATTT 360  
eGFP -----  
-----

NS1/eGFP  
GAAGGATTTAGACAGAGGAATAGACCGAGTAGAGTTGTGATGGATGATAGCTGCTCAATG 420  
eGFP -----  
-----

NS1/eGFP  
CTCTCATATTTTTACATTCCAATGAATCAAGGGAATCCAGCTCCAGTTGCGAAGCTTAGC 480  
eGFP -----  
-----

NS1/eGFP  
CGATGGGGTCAATTTGGAATTTGTTACTATGATAGAACAAATGTTGATGGATTGATTCCG 540  
eGFP -----  
-----

NS1/eGFP  
TATGATGAGATCGGTTTAGCTCAAGCTATAGACGGCCTAAAGGATCTGATTGAAGGGCGA 600  
eGFP -----  
-----

NS1/eGFP  
TTGCCCGTTTGGCCCTTATACTGGAGCGAATGGTAGAATTAATGCTGTTTTACATTTACCA 660  
eGFP -----  
-----

NS1/eGFP  
TTAGAGATGGAGGTGATTATGGCGGTGCAGGAAAAATGCAACACAATTAATGCGTAGAGCG 720  
eGFP -----  
-----

NS1/eGFP  
GCACAGGATTTTCAATTCATCACNCATGCTGGATGGAGGCTATATCCAAGATTGTTGCCGA 780  
eGFP -----  
-----

NS1/eGFP  
CAACGGTTTCGGATCGAGGACGCTACGGAGGGGGTGATTCATCATGTGATGCTAGGCCAT 840  
eGFP -----  
-----

NS1/eGFP  
TTAAGATATTATGATGAAGATAACAAGTATCGTGAAGTATCGCTTCCTTAACGATGGATCT 900  
eGFP -----  
-----

NS1/eGFP  
TTAGATTGGAGGACTTGGACAATTCCTTTACATCTGATGCGGACAGCAAGGTTGGGACAT 960  
eGFP -----  
-----

NS1/eGFP  
CTGCAACCGGAATCAATTTTAGTCTTTATGCATAAAAAGCTCACATGTCAGGTATGCTTT 1020  
eGFP -----  
-----

NS1/eGFP  
ATGGTTGACCTCGCTCTGCTTGACACAATCCCGGTGGTTGATTCAAAGATTGCTGAACTA 1080  
eGFP -----  
-----

NS1/eGFP  
ACAGGAGGCACAGATGTATTTTATACACGTGCGTATGTACATGCGGACAATCACAAAGTG 1140  
eGFP -----  
-----

NS1/eGFP  
CCAAATGTCAGAGATTTGATGATGAATGAAGTCTTCAGGAAGATCGATGATCATTGGGTG 1200  
eGFP -----  
-----

NS1/eGFP  
ATTCAGAAGTGTGCATACAACGAAGGAAGCGATTACTGTAAGTCAATTCAGATTCAGAGG 1260  
eGFP -----  
-----

NS1/eGFP  
TCGATCAGAGGTGATGGGCAGTGGGATACTCCGATGTTTCACCAATCAATGGCTCTGTTA 1320  
eGFP -----  
-----

NS1/eGFP  
ACACGATTGATTGTTTATTGGTTAACGGATGTGACTGAGAGAAGTGCTATCTTTCGGCTG 1380  
eGFP -----  
-----

NS1/eGFP  
ACTTGTTCGCAATCTTCGGATGTAAGCCAACGGCTCGAGCCATGGTGAGCAAGGGCGAG 1440  
eGFP -----  
ATGGTGAGCAAGGGCGAG 18

\*\*\*\*\*

NS1/eGFP  
GAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCAC 1500  
eGFP  
GAGCTGTTACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCAC 78

\*\*\*\*\*

NS1/eGFP  
AAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAG 1560  
eGFP  
AAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAG 138

\*\*\*\*\*

NS1/eGFP  
TTCATCTGCACCACCGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACC 1620  
eGFP  
TTCATCTGCACCACCGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACC 198

\*\*\*\*\*

NS1/eGFP  
TACGGCGTGAGTGTTCAGCCGCTACCCCGACCACATGAAGCAGCAGCTTCTTCAAG 1680  
eGFP  
TACGGCGTGAGTGTTCAGCCGCTACCCCGACCACATGAAGCAGCAGCTTCTTCAAG 258

\*\*\*\*\*

NS1/eGFP  
TCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAAC 1740



eGFP  
TCCGCCATGCCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAAC 318  
\*\*\*\*\*

NS1/eGFP  
TACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTG 1800  
eGFP  
TACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTG 378  
\*\*\*\*\*

NS1/eGFP  
AAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACACTAC 1860  
eGFP  
AAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACACTAC 438  
\*\*\*\*\*

NS1/eGFP  
AACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTC 1920  
eGFP  
AACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTC 498  
\*\*\*\*\*

NS1/eGFP  
AAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAAC 1980  
eGFP  
AAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAAC 558  
\*\*\*\*\*

NS1/eGFP  
ACCCCCATCGGCGACGGCCCCGTGCTGCTGCCGACAACCACTACCTGAGCACCCAGTCC 2040  
eGFP  
ACCCCCATCGGCGACGGCCCCGTGCTGCTGCCGACAACCACTACCTGAGCACCCAGTCC 618  
\*\*\*\*\*

NS1/eGFP  
GCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACC 2100  
eGFP  
GCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACC 678  
\*\*\*\*\*

NS1/eGFP  
GCCCGGGGATCACTCTCGGCATGGACGAGCTGTACAAGGCTCGAGGTAGATATATTGAT 2160  
eGFP  
GCCCGGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA-----  
----- 720  
\*\*\*\*\*

NS1/eGFP  
TGGGATGATCTTGAACATTCATGAAGAATGTCTTGGATGGAAGAGATTTGACTGTTTTG 2220  
eGFP  
-----  
-----

NS1/eGFP  
GAAGATGAGACATGTTTTATTTTCGATGATGAGGATGGCGATGTTGCATGTGCAGAGATCC 2280



eGFP -----  
-----

NS1/eGFP  
AAGGTAGTGTGCGCAACTGTGTTGGAGGCGCCATTAGAAAATACAACAGGTTGGCCAGATC 2340  
eGFP -----  
-----

NS1/eGFP GTTGAAGTACCCTTTGATTTTATGCATAATTAG 2373  
eGFP -----  
-----

**Appendix A-4.** CLUSTAL X (1.8) Multiple amino acid sequence alignment of AHSV6 NS1 with NS1/VP2-40 and NS1/VP2-150

```
NS1/VP2-40
MDRFLTYFQVRGERANAVRLFGEISEQIDCSHLKRDCFVNGICARQHFKECCNIATDNGS 60
NS1/VP2-150
MDRFLTYFQVRGERANAVRLFGEISEQIDCSHLKRDCFVNGICARQHFKECCNIATDNGS 60
AHSV6
MDRFLTYFQVRGERANAVRLFGEISEQIDCSHLKRDCFVNGICARQHFKECCNIATDNGS 60
```

\*\*\*\*\*

```
NS1/VP2-40
RTNADKLVALALRALLGRQTIWTCVIKNADYVSQYADEQMEEEVNRLYDVYLQSGTREEF 120
NS1/VP2-150
RTNADKLVALALRALLGRQTIWTCVIKNADYVSQYADEQMEEEVNRLYDVYLQSGTREEF 120
AHSV6
RTNADKLVALALRALLGRQTIWTCVIKNADYVSQYADEQMEEEVNRLYDVYLQSGTREEF 120
```

\*\*\*\*\*

```
NS1/VP2-40
EGFRQRNRP SRVVMDDSCSMLSIFYIIPMNQGNPAPVAKLSRWGQFGICYDR TNVDGLIP 180
NS1/VP2-150
EGFRQRNRP SRVVMDDSCSMLSIFYIIPMNQGNPAPVAKLSRWGQFGICYDR TNVDGLIP 180
AHSV6
EGFRQRNRP SRVVMDDSCSMLSIFYIIPMNQGNPAPVAKLSRWGQFGICYDR TNVDGLIP 180
```

\*\*\*\*\*

```
NS1/VP2-40
YDEIGLAQAIDGLKD LIEGR LPVCPYTGANGR INAVLHLPLEMEVIMAVQENATQLMRR A 240
NS1/VP2-150
YDEIGLAQAIDGLKD LIEGR LPVCPYTGANGR INAVLHLPLEMEVIMAVQENATQLMRR A 240
AHSV6
YDEIGLAQAIDGLKD LIEGR LPVCPYTGANGR INAVLHLPLEMEVIMAVQENATQLMRR A 240
```

\*\*\*\*\*

```
NS1/VP2-40
AQDFKFITHAGWR LYPRL LRQRFAIEDATEGVIHHVMLGHLRYDEDETSIVKYRFLNDGS 300
NS1/VP2-150
AQDFKFITHAGWR LYPRL LRQRFAIEDATEGVIHHVMLGHLRYDEDETSIVKYRFLNDGS 300
AHSV6
AQDFKFITHAGWR LYPRL LRQRFAIEDATEGVIHHVMLGHLRYDEDETSIVKYRFLNDGS 300
```

\*\*\*\*\*

```
NS1/VP2-40
LDWRTWTIPLHLMRTAR LGHLQPESILVFMHKKLTCQVCFMVDLALLDTIPVVD SKIAEL 360
NS1/VP2-150
LDWRTWTIPLHLMRTAR LGHLQPESILVFMHKKLTCQVCFMVDLALLDTIPVVD SKIAEL 360
AHSV6
LDWRTWTIPLHLMRTAR LGHLQPESILVFMHKKLTCQVCFMVDLALLDTIPVVD SKIAEL 360
```

\*\*\*\*\*

NS1/VP2-40  
TGGTDVIFYTRAYVHADNHKVPNVRDLMMNEVFRKIDDHWVIQKCHTTKEAITVTAIQIQR 420  
NS1/VP2-150  
TGGTDVIFYTRAYVHADNHKVPNVRDLMMNEVFRKIDDHWVIQKCHTTKEAITVTAIQIQR 420  
AHSV6  
TGGTDVIFYTRAYVHADNHKVPNVRDLMMNEVFRKIDDHWVIQKCHTTKEAITVTAIQIQR 420

\*\*\*\*\*

NS1/VP2-40  
SIRGDGQWDTMPFHQSMALLTRLIVYWLTDVTERSALFR LTCFAIFGCKPTARA----- 474  
NS1/VP2-150  
SIRGDGQWDTMPFHQSMALLTRLIVYWLTDVTERSALFR LTCFAIFGCKPTARAEDEELS 480  
AHSV6  
SIRGDGQWDTMPFHQSMALLTRLIVYWLTDVTERSALFR LTCFAIFGCKPTARG----- 474

\*\*\*\*\*

NS1/VP2-40 -----  
-----  
NS1/VP2-150  
REIIVAVINYGSKFGTRSGKKDLMTIDKLEKYCESLTFVHKKKRDEGDDETARAIIRN 540  
AHSV6 -----  
-----

NS1/VP2-40 -----  
LEVFKRNNKVDIDPNHDTWKNHVKEIRERM 504  
NS1/VP2-150  
QWIKGMPMSMNLKKEMKVS RGP IQNWSFFMSLEVFKRNNKVDIDPNHDTWKNHVKEIRERM 600  
AHSV6 -----  
-----

NS1/VP2-40 QKEQSANSNL-----  
ARGRYIDWDDLGTFMKNVLDGRDLTVLEDETCFIS 549  
NS1/VP2-150  
QKEQSANSNLPLKDPKLMASAYLQVARGRYIDWDDLGTFMKNVLDGRDLTVLEDETCFIS 660  
AHSV6 -----  
RYIDWDDLGTFMKNVLDGRDLTVLEDETCFIS 506

\*\*\*\*\*

NS1/VP2-40 MMRMAMLHVQRSKVVCATVLEAPLEIQQVGQIVEVPPDFMHN- 591  
NS1/VP2-150 MMRMAMLHVQRSKVVCATVLEAPLEIQQVGQIVEVPPDFMHN- 702  
AHSV6 MMRMAMLHVQRSKVVCATVLEAPLEIQQVGQIVEVPPDFMHN- 548  
\*\*\*\*\*

**Appendix A-5.** CLUSTAL X (1.8) Multiple amino acid sequence alignment of AHSV NS1 with NS1/eGFP

```

AHSV6
MDRFLTYFQVRGERANAVRLFGEISEQIDCSHLKRDCFVNGICARQHFKECCNIATDNGS 60
NS1/eGFP
MDRFLTYFQVRGERANAVRLFGEISEQIDCSHLKRDCFVNGICARQHFKECCNIATDNGS 60

*****

AHSV6
RTNADKLVALALRALLGRQTIWTCVIKNADYVSQYADEQMEEEVNRLYDVYLSQSGTREEF 120
NS1/eGFP
RTNADKLVALALRALLGRQTIWTCVIKNADYVSQYADEQMEEEVNRLYDVYLSQSGTREEF 120

*****

AHSV6
EGFRQRNRPSRVVMDSDCSMLSYFYIPMNQGNPAPVAKLSRWGQFGICYDRTNVDGLIP 180
NS1/eGFP
EGFRQRNRPSRVVMDSDCSMLSYFYIPMNQGNPAPVAKLSRWGQFGICYDRTNVDGLIP 180

*****

AHSV6
YDEIGLAQAIDGLKDLIEGRLPVCPYTGANGRINAVLHLPLEMEVIMAVQENATQLMRRA 240
NS1/eGFP
YDEIGLAQAIDGLKDLIEGRLPVCPYTGANGRINAVLHLPLEMEVIMAVQENATQLMRRA 240

*****

AHSV6
AQDFKFITHAGWRLYPRLLRQRFAIEDATEGVIHHVMLGHLRYDEDETSIVKYRFLNDGS 300
NS1/eGFP
AQDFKFITHAGWRLYPRLLRQRFAIEDATEGVIHHVMLGHLRYDEDETSIVKYRFLNDGS 300

*****

AHSV6
LDWRTWTIPLHLMRTARLGHLQPESILVFMHKKLTCQVCFMVDLALLDTIPVVDKIAEL 360
NS1/eGFP
LDWRTWTIPLHLMRTARLGHLQPESILVFMHKKLTCQVCFMVDLALLDTIPVVDKIAEL 360

*****

AHSV6
TGGTDVFYTRAYVHADNHKVPNVRDLMMNEVFRKIDDHWVIQKCHTTKEAITVTAIQIQR 420
NS1/eGFP
TGGTDVFYTRAYVHADNHKVPNVRDLMMNEVFRKIDDHWVIQKCHTTKEAITVTAIQIQR 420

*****

AHSV6
SIRGDGQWDTPMFHQSMALLTRLIVYWLTVDVTERSAIFRLTCFAIFGCKPTAR----- 473
NS1/eGFP
SIRGDGQWDTPMFHQSMALLTRLIVYWLTVDVTERSAIFRLTCFAIFGCKPTARAMVSKGE 480
*****

AHSV6
-----
-----

```

NS1/eGFP  
ELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTCLKFICTTGKLPVPWPTLVTTLT 540

AHSV6 -----  
-----  
NS1/eGFP  
YGVQCFSTRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEEDTLVNRIEL 600

AHSV6 -----  
-----  
NS1/eGFP  
KGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQN 660

AHSV6 -----  
--GRYID 478  
NS1/eGFP  
TPIGDGPVLLPDNHYLSTQSALS KDPNEKRDMVLLLEFVTAAGITLGMDELYKARGYID 720

\*\*\*\*\*

AHSV6  
WDDLGTFMKNVLDGRDLTVLEDETCFISMMRMAMLVQSKVVCATVLEAPLEIQQVGQI 538  
NS1/eGFP  
WDDLGTFMKNVLDGRDLTVLEDETCFISMMRMAMLVQSKVVCATVLEAPLEIQQVGQI 780

\*\*\*\*\*

AHSV6 VEVPFDFMHN- 548  
NS1/eGFP VEVPFDFMHN- 790  
\*\*\*\*\*