Association of nonstructural protein NS3 of African Horsesickness virus with cytotoxicity and virus virulence

By

Michelle van Niekerk

Submitted in fulfillment of the requirements for the degree

Philosophiae Doctor

In the Faculty of Natural and Agricultural Sciences (Department of Genetics)
University of Pretoria

Pretoria
November 2001

ACKNOWLEDGEMENTS

I would like to thank the following people sincerely for their contribution towards the completion of this thesis:

Dr Vida van Staden for her guidance, enthusiasm, support and supervision during the course of this study.

Prof Huismans for the opportunity he granted me to commence my PhD under his supervision and for his ongoing guidance.

Dr Albie van Dijk and her research team, Department of Biochemistry, O.V.I., for propagating the AHSV strains.

Dr Arial Körber, who has since left research to practice veterinary science, for introducing me to cell culture techniques.

Dr Janusz Paweska, Orbivirus Reference Laboratory, O.V.I. for providing the BTV strains used in this study.

Fellow colleagues in the Department of Genetics, particularly Tracy Meiring for proof reading this manuscript, for their encouragement, support and interest.

Carel Smit and Marietjie Maat for the previous construction of mutant S10 genes that were used in this study

My husband, and family for their continuous support and encouragement.

The NRF for financial support.

Dedicated to Wayne and Jolene

SUMMARY

Association of nonstructural protein NS3 of African Horsesickness Virus with cytotoxicity and virus virulence

by

Michelle van Niekerk

Promoter: Dr Vida van Staden
Department of Genetics
University of Pretoria

Co-Promotor: Prof H Huismans
Department of Genetics
University of Pretoria

for the degree PhD

Factors that determine the molecular basis of African horsesickness virus (AHSV) virulence are unclear. Several proteins and different events in the viral replication cycle together with different environmental factors are likely to contribute to the virulence phenotype. The aim of this investigation was to study the possible contribution of AHSV nonstructural protein NS3 to virus virulence. NS3 is a cytotoxic protein that localizes in areas of plasma membrane disruption and is assumed to be associated with the release of virus particles from infected cells.

A conserved feature in all AHSV NS3 proteins is the presence of two hydrophobic domains. To investigate whether these hydrophobic domains interact with membranes, cell surface localization and membrane association studies were conducted on NS3 and the two hydrophobic domain mutant forms of NS3. Results indicated that mutations in either hydrophobic domain did not prevent membrane targeting but abolished membrane anchoring. This prevented cell surface localization and obviated the cytotoxic effect of NS3.

AHSV NS3 is a highly variable protein. This level of variation may influence virulence properties. To compare the NS3 sequence variation level of South African AHSVs and bluetongue viruses (BTV/s) with those previously described, the NS3 protein sequences of field isolates, reference and vaccine strains were determined and analysed. The variation level of AHSV NS3 was found to be higher than previously reported. Comparison of the AHSV vaccine and field strain NS3 sequences revealed no obvious NS3 virulence marker. The level of AHSV NS3 sequence variation could distinguish between field isolates and vaccine strains and differentiate between sub-populations within a serotype. The inferred phylogeny of AHSV NS3 corresponded well with the described NS3 phylogenetic clusters with exception of AHSV-8 and one AHSV-6 encoded NS3. BTV NS3 inferred phylogeny indicated that the three BTV NS3 clusters that occur as geographically defined entities are all simultaneously present in S.A. This would suggest that BTV originated in southern Africa.

To investigate the impact of minor NS3 variation on AHSV virulence, the membrane permeabilization properties of AHSV-2 vaccine and virulent NS3 proteins were compared. The AHSV-2 S10 genes were cloned and NS3 was expressed using the BAC-TO-BAC system. The membrane permeabilization effect of NS3 expression was monitored by calcium influx into insect cells. Expression of either the vaccine or virulent associated AHSV-2 NS3 protein equally increased membrane permeability. The NS3 sequence variation therefor had a limited influence on membrane permeabilization properties and the virulence status of the vaccine and virulent AHSV-2 strains in this study. The possible effect of larger NS3 sequence variation levels on AHSV virulence was investigated by NS3 associated properties such as virus release and membrane permeabilization. AHSV infections increased the permeability of cell membranes and the different strains had different virus release properties. Virus release was not exclusively related to increased membrane permeability of infected cells or to the yield of the virus in the infected cells. The level of NS3 variation may influence AHSV release mechanisms and membrane permeabilization properties thereby contributing to AHSV virulence properties.

LIST OF ABBREVIATIONS

AHS	African horsesickness
AHSV	African horsesickness virus
AIDS	Acquired Immune Deficiency Syndrome
Amp	ampicillin
Amps	amperes
BHK	baby hamster kidney cells
bp	base pairs
BRDV	Broadhaven virus
BTV	bluetongue virus
°C	degrees Celcius
^{45}Ca	radiolabeled calcium
cDNA	complementary deoxyribonucleic acid
CHV	chuzan virus
Ci	Curie
CLP	core-like particle
cm^3	centimeter cubed
CMM	canine microsomal membranes
CPE	cytopathic effect
cys	cysteine
D	Dalton
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ds	double stranded
EC	endothelial cells
EDTA	ethylenediaminetetra-acetic acid
EHDV	epizootic hemorrhagic disease virus
ER	endoplasmic reticulum
et al	and others
EtBr	ethidium bromide
fcs	fetal calf serum
Fig	figure
g	gravitational force
G	gauge
gent	gentamycin
GTP	guanosine triphosphate
h	hour/s
HD	hydrophobic domain
HIV	human immunodeficiency virus
i.e.	that is
IM	intramuscular
IP_3	inosytol triphosphate
IPTG	isopropyl-β-D thiogalactopyranoside
ISA50	Incomplete Seppic adjuvant
k	kilo

kan	kanomycin
KDEL	arganine-aspartic acid-glutamic acid-luecine polypeptide
L1,L2,L3	large segments 1, 2 or 3
LB	Luaria broth
M	molar
M4,M5,M6, M7	medium segments 4,5,6 or 7
MDCK	Madin Darby bovine kidney
MEM	minimal essential medium
min	minute/s
ml	millilitre
mM	millimolar
MMOH	methyl mercuric hydroxide
MOI	multiplicity of infection
ng	nanograms
nm	nanometers
NS1, NS2,NS3	nonstructural proteins 1, 2 or 3 (refers to *Orbivirus*)
NSP4,5	nonstructural protein 4 or 5 (refers to *Rotavirus*)
OIE	*Office International des Epizooties*
OP	Onderstepoort
ORF	open reading frame
O.V.I.	Onderstepoort Veterinary Institute
P	particulate
PAGE	polyacrylamide gel electrophoresis
PAUP	phylogenetic analysis using parsimony
pBS	bluescribe plasmid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
p.i.	post infection
pmol	picomolar
PPI	peptidyl propyl isomerase
PSB	protein solvent buffer
RNA	ribonucleic acid
rNTP	ribonucleotide triphosphate
rpm	revolutions per minute
RRL	rabbit reticulocyte lysate
RT	reverse transcriptase
S	supernatant
S1 – S10	segments 1 to 10 (refers to *Orbiviruses*)
^{35}S	radiolabeled sulphur
S.A.	South Africa
SDS	sodium dodecyl sulphate
Sf9	*Spodoptera frugiperda* insect cells
SSP	single shelled particle
SRP	signal recognition particle

ss	single stranded
TE	2-amino-2-(hydroxymethly)-1,3-propanediol EDTA
tet	tetracyclin
TM	transmembrane
Tris	Tris-hydroxymethyl-aminomethane
U	units
UHQ	ultra high quality water
µg	micrograms
µl	microlitres
µm	micrometers
UN	unknown
UPGMA	unweighted pairwise geometric mean algorithm
U.S.A.	United States of America
V	volts
VIB	virus inclusion body
VLP	virus-like particle
VMP	viral membrane protein
VP1 – 7	virus protein 1 to 7
v/v	volume per volume
v/w	volume per weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
3D	three dimensional

LIST OF BUFFERS

PBS:
137mM NaCl, 2.7mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4; pH 7.3

Protein solvent buffer (PSB):
0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol

TAE:
0.04 M Tris-acetate, 0.002 M EDTA; pH 8.5

TE:
0.01 M Tris-HCl pH 7.6, 0.001 M EDTA

TGS:
0.025 M Tris-HCl pH 8.3, 0.192 M glycine, 0.1% SDS

TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Viruses have co-existed with mankind since the earliest documented times. Egyptians and other ancient civilizations recorded recognizable disease symptoms of rabies (Swanepoel, 1994), latin documents from the first and second century AD described a disease which could have been sheeppox (Fenner, 1979) and African horsesickness (AHS) was described in Yemen in 1327 (Coetzer and Erasmus, 1994).

It was only at the turn of the 20th century that scientists set out to investigate the nature of viruses. Today, more than 100 years later, mankind has greatly benefited from research in this field with the event of vaccinations and antiviral drugs. Despite ongoing research, many viral diseases still pose a challenge, one of the most renowned being human immunodeficiency virus (HIV).

Pioneer work in South Africa focused on viruses of economical importance such as bluetongue virus (BTV) and African horsesickness virus (AHSV). Sir Arnold Theiler commenced research on AHSV in the early 1900s. Of his most remarkable work was the demonstration of the plurarity of the AHSV strains, their impact on vaccination and the description of the AHS disease forms (Theiler, 1921). In the late sixties Dr Verwoerd at the Onderstepoort Veterinary Research Institute discovered that viruses such as BTV and AHSV had a segmented dsRNA genome (Verwoerd, 1969) and could be classified as genus *Orbiviridae* in the family of dsRNA viruses, *Reoviridae*. Today the field of orbivirus research has expanded to such an extent that it now includes at least 20 genera. Further advances in orbivirus research have been the elucidation of the atomic structure of the BTV core (Grimes *et al.*, 1998). Research in the other genera of the family *Reoviridae* such as the rotavirus and orthoreovirus has been more significant. A very recent breakthrough is that an infectious reovirus RNA system has been modified to generate a reovirus reverse genetics system that is potentially applicable to all dsRNA viruses (Roner & Joklik, 2001). This system could enable further studies on dsRNA virus replication and allow for the introduction of mutations into the dsRNA genome that could be valuable in the study of the structure and function of virus proteins. New approaches in vaccine development and the study of virus virulence should emerge.

1.2 THE DISEASE AFRICAN HORSESICKNESS (AHS)

1.2.1 History and current epidemiological status of AHS

African horsesickness was present in indigenous reservoir hosts when the first settlers of the Dutch East Indian Company arrived in the Cape of Good Hope in 1652 (Coetzer & Eramus, 1994). Frequent reports of the disease were subsequently recorded since the early 1700's. It was however only in 1900 that M'Fadyean transmitted AHS with a bacteria-free filtrate of blood from an infected horse suggesting that the etiological agent of AHS was viral (Coetzer & Eramus, 1994). M'Fadyean's work was confirmed by Theiler in 1901, Nocard in 1902 and Sieber in 1911. Theiler was first to suggest the plurarity of African horsesickness virus (AHSV) strains in the early 1900s which was later expanded on by Alexander and co-workers in the 1940s. To date, nine distinct strains, or serotypes, of AHSV have been described (McIntosh, 1956; Howell, 1962).

Currently AHS occurs regularly throughout subsaharan Africa and is endemic in eastern and central Africa (Coetzer & Erasmus, 1994). The disease appears every year in South Africa in the northeastern districts of the country with the extent of its southward spread being influenced by climatic conditions. In general early heavy rains followed by warm, dry spells favour the occurrence of epidemics. The onset of frost in winter usually signals the end of AHS epidemics. AHS primarily affects equine animals with horses being most susceptible while donkeys and zebras are very resistant. Donkeys in the Middle East are however more susceptible (Alexander, 1948). Seroconversion in zebra occurs every month of the year and virtually every adult animal has antibodies to all nine AHSV serotypes (Barnard, 1993). A single serotype usually dominates a particular season (Coetzer & Erasmus, 1994). Serotypes 1 to 8 are common in South Africa while serotypes 3, 4 and 9 appear to be widespread and occur outside Africa (Coetzer & Erasmus, 1994).

Pitchford and Theiler offered the possibility that AHSV was transmitted by biting insects in 1903 and in 1944 Du Toit reported that the *Culicoides imicola* midge was a vector of AHSV (Du Toit, 1944). Another *Culicoides* species, *C. botilinos* has also recently been identified as a potential vector of AHSV (Venter *et al.*, 2000). It may be possible that AHSV overwinters in the vectors as at low temperatures (< 15°C) AHSV does not replicate but may persist in some vectors at a level below that of traditional detection assays (Mellor *et al.*, 1998). When the temperature rises again virus replication is able to commence. After the virus has infected the vector and begun replication it is essential that progeny virions are deposited in the salivary ducts allowing transmission to the vertebrate hosts and this is therefor a prerequisite for vector competency (Billingsley & Lehane, 1996). As not all species of midges in the *Culicoides* genus can actively transmit AHSV it would indicate that barriers in the infection or transmission processes to salivary ducts (Jennings & Muller, 1987; Fu *et al.*, 1999) are involved and these are likely to be a result of genetically inheritable factors (Mellor, 1990). In this regard, Venter *et al.* (2000) demonstrated that experimentally infected *C. imicola* harbored AHSV-5 and –8 while *C. botilinos* harbored AHSV-3, -5 and –8.

There is no efficient treatment for AHS. Horses in southern Africa, excluding regions of the Western Cape province, are vaccinated annually with a polyvalent attenuated vaccine in an attempt to prevent disease and control outbreaks. Once the AHSV serotype involved in a specific outbreak has been identified a monovalent vaccine may be additionally administered. Other methods of control reside in good stable management and control of the vector with insecticides, repellents (Braverman & Chivoz-Ginzburg, 1998) and screens. Alternative vaccines currently being researched, such as subunit vaccines, are of interest as they are not infectious agents, and so overcome any complications associated with infectivity.

1.2.2 AHS disease forms and symptoms

AHS is considered one the most lethal of horse diseases and has been allocated *Office International des Epizooties* (OIE) List A status. Theiler distinguished four clinical forms of AHS, viz. the pulmonary form, the cardiac form, the mixed form and horsesickness fever (Theiler, 1921). The different forms of AHS (pulmonary, cardiac and fever) vary in the organs affected, severity of lesions, time of onset of clinical signs and mortality rate (Table 1.1). Erasmus (1966 & 1972) proposed that the route of infection and tropism of a sub-population of viral particles define the form of AHS seen in infected horses. The form of the disease expressed in naïve horses is a property of the AHSV inoculum (Laegreid *et al.*, 1993). No detectable virus in blood but a strong humoral response of animals with the mild fever form of AHS suggest that impaired secondary spread may be involved in the differing virulence (Laegreid *et al.*, 1993)

1.2.3 Pathogenesis of AHS

Three diseases caused by members of the genus *Orbivirus* are of economic importance. These include bluetongue, epizootic hemorrhagic disease and African horsesickness. Bluetongue causes major loss to the sheep and cattle farming industry. Epizootic hemorrhagic disease (EHDV) is a fatal disease of white deer that also induces clinical and pathological signs similar to bluetongue in cattle and other ruminants (Metcalf & Luedke, 1980). These diseases share similar clinical symptoms in infected animals including hemorrhages and general fluid imbalances. Rotavirus, a related virus in the same family (*Reoviridae*) also causes fluid imbalances in infected cells. The types of cells that are susceptible to the effects of the virus differ to some extent from virus to virus. Rotavirus has a drastic effect on intestinal cells while the orbiviral diseases affect endothelial cells with bluetongue specifically affecting the tongue and African horsesickness the lungs and spleen. Bluetongue is characterized by pathological features including inflammation, hemorrhage, ulceration, cyanosis of the mucous

membrane of the oronasal cavity, coronitis, laminitis and oedema of the head and neck (Verwoerd & Erasmus, 1994). BTV initially replicates in lymph nodes before the virus dessiminates to the lungs and spleen where it replicates in endothelium and mononuclear phagocytes (MacLachan *et al.*, 1990). BTV replicates in monocytes and replicating lymphocytes but the infection does not progress beyond adsorption in erythrocytes and non-replicating lymphocytes of bovine blood cells were the virus particles persist in invaginations of the cell membrane (Brewer & MacLachlan, 1994). The interaction of BTV with erythrocyte cell membranes in infected cattle may facilitate prolonged viremia as well as infection of the insect vector (MacLachlan, 1994). BTV could be isolated from most tissue of infected calves but no virus could be detected upon termination of viremia (Barrat-Boyes & Maclachlan, 1994). No distinct forms of the bluetongue have been described and the course of the disease in sheep can vary from peracute to chronic, with a mortality rate of between 2 and 30% (Verwoerd & Erasmus, 1994) while infection in cattle is asymptomatic with prolonged viremia. In AHSV infections hemorrhages are much less severe and thrombi are not observed as seen with EHDV infections.

The pathogenesis of AHS is not well understood. A variety of factors may influence viral pathogenesis including viral tropism, permissivity, host immune status and host genetic susceptibility (Laegried *et al.*, 1993). The prominent pathological features of AHS (oedema, effusion and hemorrhage) suggest loss of endothelial cell (EC) barrier function as an inciting cause (Skowronek *et al.*, 1995). Pathogenesis of fatal AHS involves injury to ECs leading to an increase in vascular permeability and activation of the coagulation and fibronolytic systems. Injury to the endothelium of small blood vessels, possibly in combination with the hosts' inflammatory reaction, is responsible for the manifestation of disease in BTV-infected sheep (Demaula *et al.*, 2001).

AHSV infects ECs and alters their fluid balance mechanisms (Laegreid *et al.*, 1992a). The presence of AHSV particles and ultrastructure damage to pulmonary

vascular ECs provides morphologic support for the view that the clinical signs and lesions of AHS may be related to the ability of the virus to infect ECs. Virulence variants of AHSV differ in their ability to infect EC (Laegreid *et al.*, 1992b). ECs differ in many respects depending on the size, type and tissue of origin of the vessel from which they are derived. Clinical symptoms elicited by an *in vitro* infection of ECs from distinct anatomic sites with a given AHSV isolate is a function of the isolate's ability to infect populations of ECs (Laegreid *et al.*, 1992b). Serial passage of AHSV in horses using lung material consistently produced peracute disease with marked pulmonary involvement, whereas passage using spleen material resulted in progressively milder diseases (Newsholme, 1983). Pronounced cell swelling with some loss of integrity of the plasma membrane is observed in the most severely affected cells.

Multiple mechanisms may be involved in the development of pulmonary microvascular leakage. A study conducted with ECs infected with an AHSV-4 strain showed multiple foci of loss of structural detail and discontinuity of the plasma membrane (Laegreid *et al.*, 1992b). Distinct loss of integrity and intercellular junctions along with severe damage to ECs themselves indicates a loss of EC barrier function, a defect that results in oedema (Laegreid *et al.*, 1992b).

Oedema in tissue may be classified into permeability, hydrostatic and lymphatic types (Hurley, 1978). The abundance of fibrin observed in alveoli and pulmonary interstitial tissue in the pulmonary form of AHS suggests that the oedema fluid has a high protein content compatible with that of permeability oedema (Newsholme, 1983). Severe pulmonary oedema of the permeability type can result from minor, reversible structural changes in endothelium (Newsholme, 1983). Widespread evidence of interstitial oedema and occurrence of interstitial fibrin, both observed at capillary level, together suggest that capillaries are an important site of increased permeability in AHS. Separation of capillary

Table 1.1 Summary of AHS disease forms (compiled from Erasmus, 1972; Laegreid *et al.*, 1993; Maurer & McGully, 1963; Coetzer & Erasmus, 1994)

	Disease form			
	Pulmonary	**Cardiac**	**Fever**	**Mixed**
Symptoms	Severe pulmonary oedema, hydrothorax oedema, exudative pneumonia	Pericardial effusion, sub-endocardial hemorrhage, sub-cutaneous oedema, hydropericardium	Transient mild fever	Combination of pulmonary & cardiac
Affected Organs	Lungs, hydrothorax, bronchial and mediastinal nodes, spleen. High virus titre in blood early in infection that increases dramatically in 2 days and plateaus for 3-5 days before death	Subcutaneous & intermuscular tissue of head & neck, heart, liver. Virus in blood 7 days p.i. that increases but drops to 0 by day 16 p.i.	No detectable virus in blood but a strong humoral response.	Mixture of pulmonary & cardiac
Histopath-ology	Alveolar & interstitial oedema (inflammatory origin), oedema fluid contains inflammatory cells & sometimes fibrin	Myocardial oedema & hemorrhage, sometimes myocarditis		
Incubation period	4-5 days	Variable, 7-14 days	5-14 days	5-7 days
Mortality Rate	Approaches 100%	70%	0%	> 80%

endothelial intercellular junctions may also constitute a major pathway of fluid leakage in AHS (Newsholme, 1983).

1.3 AFRICAN HORSESICKNESS VIRUS TAXONOMIC CLASSIFICATION

AHSV is a member of the family *Reoviridae*, genus *Orbivirus*. The nine genera of the *Reoviridae* include viruses that have segmented double stranded (ds) RNA genomes (10 - 12 segments), are icosahedral in shape (55 - 80 nm) and have a broad host range including plants, vertebrates and invertebrates. The virus particles have an inner protein capsid, and one (or two) distinctive outer capsids (Urbano & Urbano, 1994).

Members of the genus *Orbivirus*, the largest genus within the family *Reoviridae*, are subdivided into 19 distinct serogroups (Table 1.2) that consist of 149 serotypes. Twenty-four serotypes have been described for bluetongue virus (BTV), the prototype orbivirus. The AHSV serogroup includes 9 virus serotypes that are differentiated by neutralization studies of the major outer capsid viral protein, VP2 (McIntosh, 1956; Howell, 1962). Nearly all orbiviruses have been isolated from anthropods and many have been isolated from vertebrates (Calisher & Mertens, 1998).

1.4 AFRICAN HORSESICKNESS VIRUS STRUCTURE

AHSV is comparable to BTV in both morphology and molecular constitution (Roy *et al.*, 1994). These viruses have seven structural proteins (VP1 - 7) organized in a double layered capsid (Bremer, 1976; Huismans, 1979; Mecham & Dean, 1988). The outer capsid is composed of VP2 and VP5 while the inner capsid is composed of two major proteins, VP3 and VP7, and three minor proteins, VP1, VP4 and VP6. In addition to the seven structural proteins, at least four nonstructural proteins NS1, NS2 and NS3 and NS3A are encoded.

Table 1.2 Viruses of the genus *Orbivirus* (Calisher & Mertens, 1998)

Virus Group	**Number of Sero-types**	**Hosts**	**Principle Vector**
AHSV	9	Equids, dogs, elephants, camels	*Culicoides*
BTV	24	Cattle, sheep, elephants	*Culicoides*
Changuinola virus (CGLV)	12	Humans, rodents, sloths	Phlebotomine flies, mosquitoes
Chenuda virus (CNUV)	7	Seabirds	Ticks
Chobar Gorge virus (CGV)	2	Bats	Ticks
Corriparta virus (CORV)	3	Humans, rodents	Culicine mosquitoes
Epizootic haemorrhage disease virus (EHDV)	18	Cattle, sheep, deer, camels, lamas	*Culicoides*
Equine encephalosis virus (EEV)	7	Equids	*Culicoides*
Eubenangee virus (EUBV)	4	Unknown	*Culicoides*, Culicine mosquitoes
Ieri virus (IERI)	3	Birds	*Culex* mosquitoes
Great Island virus (GIV)	36	Seabirds, rodents, humans	Ticks
Lebombo virus (LEBV)	1	Humans, rodents	Culicine mosquitoes
Orungo virus (ORUV)	4	Humans, camels, cattle, goats, sheep, monkeys	Culicine mosquitoes
Palyam virus (PALV)	11	Cattle, sheep	*Culicoides*, Culicine mosquitoes
St Croix River virus (SCRV)*	?	Deer	Ticks
Umatilla virus (UMAV)	4	Birds	Culicine mosquitoes
Wad Medani virus (WMV)	2	Domestic animals	Ticks
Wallal virus (WALV)	2	Marsupials	*Culicoides*
Warrego virus (WARV)	2	Marsupials	*Culicoides*, Anopheline, Culicine mosquitoes
Wongorr virus (WGRV)	8	Cattle, macropods	*Culicoides*, Culicine mosquitoes

* SCRV is a recent addition to the genus orbivirus (Attoui *et al.*, 2001)

The genome within the inner capsid consists of 10 dsRNA segments of different sizes designated L1-L3, M4-M7 and S8-S10 in order of decreasing size where L, M and S imply large, medium and small respectively. The 5' non-coding regions of the dsRNA genomic segments range in length from 12 base pairs (bp) to 35 bp, while the 3' non-coding regions are longer, ranging from 29 bp to 100 bp (Roy *et al.*, 1994). Unlike BTV RNA segments, the 5' and 3' terminal hexanucleotides of AHSV RNA segments are not conserved throughout all the segments. The 5' and 3' end sequences of each AHSV RNA segment show partial inverted complementary, indicating that they may be capable of forming secondary structures for the single stranded (ss) RNA species.

1.4.1 Structural proteins

1.4.1.i Outer capsid proteins

VP2 with VP5 compose the outer capsid of the AHSV virion (Roy *et al.*, 1994). VP2 (encoded by L2) is the most variable AHSV protein (approximately 64% variation on amino acid level across the serotypes). It is the major outer capsid protein and contains the neutralizing epitopes of AHSV (Barrage *et al.*, 1993). A large number of the antigenic determinants are located in the amino terminal half of VP2 (Bentley *et al.*, 2000). Genetic reassortment of L2 between virulent and avirulent phenotypes of AHSV isolates indicates that VP2 may influence the virulence phenotype of progeny virus isolates (O' Hara *et al.*, 1998).

VP5 (encoded by M6) is more conserved amongst the AHSV serotypes (19% variation on amino acid level between different serotypes). VP5 is mostly insoluble when expressed in insect cells, is present in small amounts in infected cells and appears to be cytotoxic for infected Sf9 cells (Roy *et al.*, 1994). O'Hara and co-workers (1998) also indicated a possible role for VP5 in the virulence phenotype expressed by a given isolate. Purified VP5 is able to permeabilize

mammalian and *Culicoides* cell membranes, inducing cytotoxicity (Hassan *et al.*, 2001). VP5 of BTV has been shown to be glycosylated (Yang & Li, 1993)

1.4.1.ii Inner capsid proteins

VP3 and VP7, encoded by L3 and S7 respectively, are the two major inner capsid proteins. These proteins are highly conserved amongst the serotypes and contain the group-specific antigenic determinants. Co-expression of VP7 and VP3 results in their aggregation and forming of empty core-like particle (CLP) structures (French & Roy, 1990; Maree *et al.*, 1998). The inner capsid is arranged in clusters of VP7 trimers on top of a shell of VP3 molecules (Hewat *et al.*, 1992; Prasad *et al.*, 1992). VP7 is the major component of the inner capsid and forms hexagonal crystalline structures (250 μm in length) when expressed by a baculovirus recombinant in Sf9 cells (Roy *et al.*, 1994). The RGD motif between amino acids 168 – 170 of BTV VP7 is responsible for cell binding activity to *Culicoides* cells (Tan *et al.*, 2001)

VP1, encoded by L1, is considered to be the virus replicase-transcriptase and is presumed to be capable of elongating RNA when supplied with a single stranded substrate as proposed for BTV VP1 (Roy *et al.*, 1994). VP4 of AHSV, encoded by M4 is likely to have analogous functions to BTV VP4, a gaunyl transferase that methylates mRNA's during transcription (Roy *et al.*, 1994). BTV VP6 is believed to possess helicase activity to unwind the dsRNA genome prior to synthesis of complementary RNA and has a concentration dependant ss- and dsRNA binding activity (Hayama & Li, 1994). AHSV VP6 has a degree of sequence similarity to a helicase of *E. coli* (Turnbull *et al.*, 1996).

1.4.2 Nonstructural proteins

The M5 dsRNA segment of AHSV encodes the nonstructural protein NS1 that is highly conserved throughout AHSV serotypes. NS1 is rich in cysteine residues (9 out of the 16 are conserved in the same position of AHSV and BTV NS1) indicating the existence of a highly ordered and conserved structure in the protein (Roy *et al.*, 1994). Virus-specific tubules observed in AHSV infected cells are composed of helically coiled ribbons of NS1 dimers. The conserved cysteine residues at positions 337 and 340 are essential for BTV NS1 tubule formation and may be as important for AHSV NS1. Despite the high level of sequence conservation, AHSV NS1 has a lower sedimentatioin rate than that of BTV NS1 and does not possess the same ladder-like structure (Maree & Huismans, 1997). The function of NS1 is unknown.

AHSV NS2 is encoded by the S8 dsRNA segment and is the only viral phosphorylated protein in infected cells. NS2 is a major component of virus inclusion bodies (VIBs), dense granular bodies, routinely observed in the cytoplasm of AHSV infected cells and are the sites of virus assembly. AHSV NS2 is also highly conserved with the termini of the protein being rich in hydrophobic domains and β-turns. NS2 has the ability to bind ssRNA, a property shared with NS2 of BTV, δ NS protein of reoviruses and the NSP5 protein of rotaviruses. The RNA binding activity of NS2 probably indicates that it is involved in recruiting mRNA during the virus assembly process. NS2 is phosphorylated on certain serine residues (Devaney *et al.*, 1988) and it has been found that unphosphorylated EHDV NS2 binds ssRNA more efficiently than the phosphorylated version (Theron *et al.*, 1994). BTV NS2 has been found to possess the greatest affinity for binding ssRNA, followed by that of EHDV and then AHSV NS2 (Uitenweerde *et at.*, 1995).

The S10 segment of AHSV encodes two related nonstructural proteins in the same reading frame from alternate start codons, namely NS3 and NS3A (Van

Staden & Huismans, 1991; Mertens *et al.*, 1984; Van Dijk & Huismans, 1988). Unlike the other AHSV nonstructural proteins NS1 and NS2, AHSV NS3 is highly variable. Despite the high level of sequence variation, AHSV NS3 shares numerous conserved features with NS3 proteins of other orbiviruses. These include (Van Staden *et al.*, 1998); two in phase methionine start codons (approximately 11 amino acids apart), a proline rich region (residues 22 - 34), a highly conserved domain between amino acids 43 and 93 and two hydrophobic domains between residues 116 – 137 and 154 –176.

The hydrophobic domains may form putative transmembrane (TM) domains. Proposed models based on computational modeling predict two alternative membrane-spanning conformations for NS3. Both hydrophobic regions could span the membrane with the terminal regions located in the cytoplasm (Van Staden *et al.*, 1995). Alternatively, a similar conformation could be adopted to that determined for rotavirus NSP4 whereby only the second domain spans the membrane with the amino terminal orientated in the endoplasmic reticulum (ER) lumen (Jensen & Wilson, 1995). AHSV NS3 is cytotoxic when expressed in the baculovirus expression system in Sf9 (*Spodoptera frugiperda*) cells (Van Staden *et al.*, 1998). Substitution mutations in either hydrophobic domain of AHSV NS3 were found to abrogate its cytotoxic effect in Sf9 cells (Stoltz, 1994; Smit, 1999). Other substitution mutations directed towards the proline rich and highly conserved regions had little effect on NS3 induced cytotoxicty as did a deletion mutation that forced exclusive expression of NS3A in Sf9 cells (Smit, 1999). No evidence suggests that AHSV NS3 is glycosylated despite the presence of glycosylation motifs. AHSV NS3 is expressed in small amounts in infected mammalian or Sf9 cells.

A novel feature of AHSV NS3 expression in a baculovirus system is its cytotoxic effect on Sf9 cells. Only 5% of a Sf9 cell population harboring NS3 was found to be viable 48 hours post infection (Van Staden *et al.*, 1995). This unique characteristic of NS3 has raised the question of its involvement in other virus

attributes such as pathogenicity and virulence, features that are not necessarily serotype correlated. The connection between NS3 and virulence has been demonstrated in genomic reassortment studies between virulent and avirulent strains of AHSV using a mouse model. O'Hara *et al.* (1998) showed that the exchange of the NS3 gene influenced virulence characteristics of the progeny viruses. One possible explanation for the difference in virulence characteristics was the different timing of virus release from infected cells (Martin *et al.,* 1998). NS3 is present in membrane components of infected cells, specifically localized to sites of AHSV release from Vero cells (Stoltz *et al.,* 1996). This suggests the involvement of NS3 in the final stages of viral morphogenesis and release, similar to that previously proposed for the homologous NS3 protein of BTV (Hyatt *et al.*, 1993). This release can occur prior to visible cytopathic effect (CPE) or cell lysis. AHSV NS3 expressed by recombinant baculoviruses is plasma membrane associated, indicating that its localization is not dependent on the presence of AHSV particles (Stoltz *et al.,* 1996).

NS3 (and therefor also NS3A) can significantly influence vector competence and therefor transmission of AHSV (Riegler *et al.*, 2000). The midgut of midges is infected after ingestion of a blood meal from a viremic mammalian host. AHSV then replicates and is disseminated throughout the midge, infects the salivary glands and can be re-transmitted through a subsequent feeding (Riegler *et al.*, 2000). Besides influencing efficient virus dispersion, NS3 possibly contributes to other virus-induced changes in host cell metabolism and viability. Many other viruses result in similar induced changes due to the expression of small membrane associated proteins commonly termed viroporins. Viroporins induce membrane damage that can range from gross membrane disruption to slight alterations in membrane permeability of infected cells. Changes in membrane permeability directly influences cellular metabolism (feedback inhibition, second messenger cascades, pH etc.) and therefor also cell viability.

Although S10 sequence data of other orbiviruses such as CHV, EDHV and BRDV is known only BTV S10 and the encoding NS3 protein have been further studied in terms of structure/function relations. Another well-characterized membrane associated nonstructural protein is NSP4, the cognate rotavirus protein to NS3. As NS3 is the focus of this thesis it was considered necessary to look at these research fields in more detail.

1.5 NS3 OF BLUETONGUE VIRUS AND THE ANALOGOUS PROTEIN IN ROTAVIRUS, NSP4

1.5.1 Bluetongue virus encoded NS3

BTV is the prototype orbivirus and thus information gathered for BTV directly relates to AHSV. BTV encodes two related proteins NS3 and NS3A from the same reading frame of genome segment S10 (Van Dijk & Huismans, 1988), as found in AHSV. However, unlike AHSV NS3, BTV NS3 sequence is highly conserved amongst BTV serotypes (Hwang *et al.*, 1992*a*). NS3 and NS3A expressed in the baculovirus system reacted with sera from sheep infected with homologous and heterologous BTV serotypes, suggesting that BTV S10 gene products are highly conserved group specific antigens (French *et al.*, 1989). Comparative analysis of BTV NS3 proteins revealed a single conserved tyrosine residue as position 159, two conserved cysteine residues (137 & 181), two N-linked glycosylation sites (63-65 & 150-152) and a cluster of six proline residues within a 15 amino acid region near the amino terminus (Hwang *et al.*, 1992*a*). BTV NS3 is estimated to be expressed at approximately 10μg per 10^6 cells in the baculovirus system while only very small amounts are present in BHK infected cells (French *et al.*, 1989). NS3A is expressed in smaller amounts than NS3 in the baculovirus system and usually requires [^{35}S] pulse labeling for detection (French *et al.*, 1989). The adaption of BTV to grow in mosquito cells (C6/36)

resulted in high level expression of NS3/A and subsequent release of BTV particles (Guirakhoo *et al.*, 1995).

NS3 and NS3A were observed in the plasma membrane of infected cells associated with areas of membrane perturbation (Hyatt *et al.*, 1991). This suggested that these proteins are involved in the final stages of BTV morphogenesis. The first hydrophobic region seemed important for introducing the protein into the ER to allow subsequent glycosylation (Bansal *et al.*, 1998). NS3/NS3A mediated release of virus-like particles (VLPs), but not CLPs from infected cells (Hyatt *et al.*, 1993). Glycosylated NS3 and NS3A are detected as high molecular weight forms in mammalian cells (Wu *et al.*, 1992). Both glycosylated and unglycosylated forms are localized in the Golgi complex as integral membrane proteins (Wu *et al.*, 1992). Glycosylation most likely protects the protein from degradation either enroute to incorporation into the cell surface or thereafter (Bansal *et al.*, 1998). It has been suggested that both the hydrophobic regions of BTV NS3 span the cell membrane and only the site at amino acid 150 is glycosylated (Bansal *et al.*, 1998).

1.5.2 Rotavirus encoded NSP4

Rotavirus, another member of the family *Reoviridae,* has been comprehensively studied and has served as models and provided guidelines for many investigations on related dsRNA viruses. As previously mentioned rotavirus infections cause fluid imbalances in infected cells, a characteristics common to AHSV infections with the causative agent being NSP4, the cognate protein to NS3 of *Orbiviruses*. The role of NSP4 can be divided into two distinct sections, the first involving viral morphogenesis and the second involving the effect of NSP4 on infected cells. Based on these guidelines, investigations have been conducted on the orbivirus NS3 protein to elucidate the function of NS3.

NSP4 is a nonstructural TM, ER-specific glycoprotein. Calnexin, a molecular chaperone, interacts with and retains NSP4 within the ER (Mirazimi *et al.*, 1998) NSP4 assembles into homotetramers mediated by the cytoplasmic region (residues 95 -137) that adopts a α-helical coiled-coil structure (Maass & Atkinson, 1990; Taylor *et al.*, 1996). These NSP4 oligomers form virus-binding domains that serve as intracellular receptors for single-shelled rotavirus particles (SSP) and assist in their translocation across the ER membrane. VP6, the major structural protein on the surface of SSPs, constitutes the ligand in the initial interaction with NSP4 (Au *et al.*, 1989). Integral membrane status is not an essential prerequisite for ligand binding (Taylor *et al.*, 1993). The region between amino acids 161 and 175 of NSP4 binds SSP and is dependent on the integrity of the cytoplasmic C-terminal (Au *et al.*, 1993; O'Brien *et al.*, 2000). Deletion or conservative substitution of the C-terminal methionine completely abolishes receptor activity (Taylor *et al.*, 1992). Glycosylation of NSP4 and Ca^{2+} play a major role in the assembly and acquisition of specific viral protein conformations necessary for the correct association of proteins during virus maturation in the ER (Poruchynsky *et al.*, 1991). NSP4 glycosylation is required for the removal of the transient envelope from budding virus particles following translocation (Petrie *et al.*, 1983). In addition to binding VP6, NSP4 binds VP4, an outer capsid protein, between residues 112 and 148 (Mattion *et al.*, 1994).

In conjunction with other rotavirus proteins (VP3, VP4 and VP7) NSP4 plays a role in determining the viral pathogenecity in humans (Kirkwood *et al.*, 1997) and in gnotobiotic piglets (Hoshino *et al.*, 1995). Free NSP4 or a 22 amino acid NSP4 peptide (residues 114 -135) induces diarrhea when administered to 6 - 10 day old mice (Ball *et al.*, 1996). Structural changes between amino acid 131 and 140 also play a role in pathogenecity by influencing biological activities relating to intracellular calcium levels and avirulence can be associated with mutations in this region (Zhang *et al.*, 1998). Ca^{2+} is important for rotavirus replication and cytopathogenesis (Ruiz *et al.*, 2000). The physical integrity of rotavirus particles requires Ca^{2+} as does the oligomerization of NSP4 and other rotavirus proteins.

A significant increase in the free cytosolic Ca^{2+} concentration in rotavirus infected MA104 cells is related to the cytotoxicity observed in infected cells (Michelangeli *et al.*, 1991). Cell death of rotavirus infected MA104 cells, attributed to an increase in the intracellular Ca^{2+} concentration, is related to a product of viral protein synthesis rather than to the production of newly formed virions (Perez *et al.*, 1998). NSP4 expression in Sf9 cells causes an increase in the internal Ca^{2+} concentration (Tian *et al.*, 1994) by increasing the release of Ca^{2+} from the ER (Tian *et al.*, 1995). Exogenous NSP4 or the 114 -135 NSP4 peptide also increases the internal cytosolic Ca^{2+} concentration through the activation of phospholipase C (Tian *et al.*, 1995). Addition of purified NSP4 to a human intestine cell model mobilized Ca^{2+} from a subset of neurotensin-sensitive Ca^{2+} pools related to both an internal Ca^{2+} store release and Ca^{2+} specific influx (Dong *et al.*, 1997) and it seems likely that NSP4 has a cell surface receptor. Their results suggest that NSP4 induces early diarrhea by promoting Ca^{2+}- dependant fluid secretion across the mucosa and not by an ultrastructural change in mucosal integrity. NSP4 has been documented to be the first viral enterotoxin. The enterotoxin active region has been mapped to residues 112-175. This domain is actively secreted from cells by the microtubule and actin microfilament network (Zhang *et al.*, 2000) This further provides evidence that NSP4 is responsible for some aspects of rotavirus virulence. Increase in intracellular calcium concentration is responsible for cell lysis and release of mature virus particles during the late stage of virus replication cycle (Ruiz *et al.*, 2000). Purified NSP4 releases calcein (fluorescent marker) incorporated into liposomes and breaks up microsomes as does the NSP4 114-135 peptide (Tian *et al.*, 1996). This suggests that NSP4 has membrane destabilizing activity and causes ER damage. NSP4 expressed in MA104 cells also results in a loss of plasma membrane integrity and morphological analysis reveals gross changes to cell ultrastructure, indicative of cell death (Newton *et al.*, 1997). Newton *et al.* (1997) further describes the importance of an α-helical wheel proximal to the membrane spanning region in the cytoplasmic domain that may mediate cytotoxicity.

Like BTV NS3 and rotavirus NSP4, AHSV NS3 has been shown to be membrane associated. AHSV NS3 is also cytotoxic, a characteristic it shares with NSP4. These common features are likely to play an important part in the contribution of these proteins to viral pathogenesis and it was considered relevant to discuss these topics in more detail.

1.6 BIOLOGY OF VIRAL MEMBRANE PROTEINS (VMPS)

Many viral membrane proteins comprise complex high molecular weight transmembrane oligomers that undergo numerous post-translational modifications. Functions of VMPs include (Doms *et al.*, 1993) viral binding to receptors on host cell plasma membrane, mediation of membrane fusion, virus morphogenesis and budding, receptor destroying enzymes necessary for virus release and ion channel formation, in particular by the small molecular weight proteins. AHSV NS3 does not constitute any structural component of the virion and is therefor unlikely to be involved in viral binding and mediation of membrane fusion in the infection process. However, it may be involved with virus morphogenesis, virus release and ion channel formation. As many functions of membrane related proteins are ascribed to their specific cellular localization, the mechanism of membrane targeting and insertion of small viral membrane proteins will be further discussed.

1.6.1 Folding and assembly of VMPs

Folding and conformation maturation are not spontaneous events but are assisted by folding enzymes and molecular chaperones. The ER maintains high calcium concentrations, oxidizing redox potential (disulphide bonds), ATP and associated chaperones and provides a specialized high capacity folding environment in which polypeptides can efficiently attain 3-D structures. An increasingly complex macromolecular transport machine, the translocon, is

responsible for the proper transport and biogenesis of proteins at the ER membrane (Walter & Lingappa, 1986). Membrane protein biogenesis is speculated to take place with a releasably bound ribosome to the translocon. The translocon consists of a heterotrimeric Sec61 complex that is adjacent to translocating nascent chains and translocating-chain associated membrane protein (TRAM) (Hedge & Lingappa, 1997). The translocon facilitates the movement of secretory proteins across the ER membrane while it recognizes potential membrane-spanning domains facilitating their proper insertion and orientation in the plasma membrane.

The topological domains of VMP fold under different conditions and constitute independent folding domains within the viral proteins (Doms *et al.*, 1993). The ectodomain contains carbohydrate moieties or disulphide bonds and folds in the lumen of the ER similar to that of secretory and membrane glycoproteins. The transmembrane domain/s adopt an α-helical configuration in the hydrophobic bilayer. The cytoplasmic region usually constitutes the bulk of the protein and follows folding rules similar to cytosolic proteins. The primary sequence is critical. Chaperones and folding enzymes are also important and prevent irreversible aggregation, catalyze rate limiting steps (disulphide bond formation and proline isomerization) and increase efficacy. Folding rates differ from protein to protein. Defective or misfolded proteins destined for membranes are retained in the ER or in other early compartments where they accumulate and are repaired or eventually degraded (Lippincott-Schwartz *et al.*, 1988). Correctly folded proteins move to the Golgi for further processing, such as carbohydrate addition or cleavage. Folding enzymes include protein disulphide isomerase (PDI), peptidyl propyl isomerase (PPI) and chaperones such as GRP78-BiP. Glycosylation may play an important role in folding but probably increases protein solubility rather than having a local function in the folding process.

Formation of oligomers may be as result of disulphide bond formation or, more common, non-covalent interactions. Oligomerization occurs in the ER or during

transit to the cis-Golgi. Assembly is not inhibited by a decrease in temperature, energy deprivation or Brefeldin A treatment all of which block ER to Golgi transport. Coiled-coiled domains of proteins often stabilize the protein and allow for protein dimerization as the α-helical coils wind around each other (Bassel-Duby *et al.*, 1985). Polar and electrostatic interactions are implicated to be critical in the assembly process. A principle governing the assembly of bacterial inner membrane proteins and a large number of eukaryotic proteins is that peripheral segments containing excess positive charges do not tend to cross the lipid bilayer (Cramer *et al.*, 1992).

1.6.2 Targeting and insertion of membrane associated proteins

Membrane proteins can adopt different morphological orientations in cellular membranes. Type I integral membrane proteins are most common and expose their N-terminal domain extracellularly while type II integral membrane proteins have their C-terminal exposed extracellularly. It is further possible that both protein terminals are exposed intracellularly.

Proteins targeted and inserted into the ER by a signal-anchor sequence associate with the 54 kD signal recognition particle and numerous other components. Ribosome, signal recognition particle (SRP), GTP and rough ER are required for targeting and insertion of type I signal-anchor proteins (High *et al.*, 1991). A minimum stretch of 22 amino acids is required to span the phospholipid bilayer as an α-helix. These helices could be composed of hydrophobic residues or could be amphipathic with its nonpolar face interacting with the hydrophobic fatty acyl side chains (Lodish, 1988). Multiple-spanning membrane proteins may consist of outer and inner helices. The inner helices need not be 22 amino acids in length (Lodish, 1988).

To demonstrate the diversity and uniqueness of membrane targeting and insertion signals of viral membrane proteins various examples found in the literature will be discussed briefly.

Cellular localization of polyoma virus middle T antigen is regulated by at least two determinants, a transmembrane sequence which confers membrane binding and a basic motif which specifies a particular site within the membrane (Elliot *et al.*, 1998). It was found that the transmembrane domain of rubella virus E2 envelope glycoprotein and the cytoplasmic domain of rubella virus E1 are crucial for early and late steps respectively in the rubella viral assembly pathway and virus morphogenesis (Garbutt *et al.*, 1999). E2 envelope glycoprotein contains a Golgi retention signal in its transmembrane domain, whereas a signal for ER retention of E1 has been localized to its the transmembrane and cytoplasmic domains. In the herpes simplex glycoprotein gD and adenovirus integral membrane protein E3-11.6 K the membrane anchoring and the cytoplasmic domains are necessary for localization in the nuclear envelope and could influence retention in the ER and Golgi complex (Ghosh & Ghosh, 1999).

Poliovirus 2C protein, required for viral replication and possibly for virion assembly and structure (Li & Baltimore, 1990), is localized to the ER despite the lack of a significant hydrophobic domain (Cho *et al.*, 1994) or ER targeting and retention signals (such as C-terminal sequence KDEL or a lysine rich region) (Paabo *et al.*, 1987; Pelham & Munro, 1993). The N-terminal region contains a putative amphipathic helix (residues 21 - 54) that has been shown to play an important role in membrane binding *in vivo* and *in vitro* (Echeverri & Dasgupta, 1995). Protein 2C is not glycosylated nor is it cleaved by signal peptidase. Hepatitis C virus (HCV) is a member of the genus *Flavivirus* that buds from the ER. E1gp of HCV is a type I integral membrane protein and is N-linked glycosylated. E1gp localizes to the ER and is retained there despite the absence of typical retention signals. The retention signal is likely to be associated with the N terminal of E1gp as fusion of this region to the ectodomain of CD4 prevents it

from being transported to the cell surface (Flint & McKeating, 1999). The TM domain of E1gp further has membrane-active properties that may be involved in some aspects of virus-cell interaction (Ciccaglione *et al.*, 1998). Protein E2gp is also retained in the ER. C-terminal truncation of the protein (residues 661 - 715) leads to secretion, consistent with deletion of a proposed hydrophobic TM anchor sequence (Flint *et al.*, 1999). Interestingly, mutations in the middle of the TM domain of influenza virus hemaglutinin was found to reverse the polarity of its transport from the apical cell surface to the basolateral surface of certain epithelial cells (Lin *et al.*, 1998).

As can be seen from the above mentioned examples numerous signals exist for membrane protein targeting and insertion. The signals are diverse in nature and seem to be virus-specific.

1.7 MODIFICATION OF MEMBRANE PERMEABILITY BY ANIMAL VIRUSES

Cytolytic viruses are known to cause the host cells to disintegrate by increasing plasma membrane permeability, causing a loss of cellular ion gradients and leaking of essential compounds from the cell (Carrasco, 1994) that leads to necrosis. Animal viruses are known to permeabilize cells both early during the infection of cells as well as during expression of a virus gene encoding ionophores or membrane active toxins.

Activity of the membrane active toxins may be related to virus interference with host cell metabolism and the cytopathic effect (CPE) that develops after virus infection. Early membrane permeabilization promotes co-entry of macromolecules such as protein toxins into the cell and has been used to introduce plasmids in tissue culture (Carrasco, 1994). Since AHSV NS3 is membrane associated and related to virus virulence, the most obvious

mechanism responsible for its cytotoxicity is likely to be associated with changes in membrane permeability.

1.7.1 Viroporins

The specific phenomenology of plasma membrane permeabilization is a characteristic of not only the animal virus but also the cell it infects. Viroporins are small virus encoded proteins that form pores in the membrane, affect cellular macromolecule synthesis and destabilize the membrane (Carrasco, 1994). In some viruses a single gene product is the major cause of the CPE. The virus products involved in cytopathicity most probably have the plasma membrane as primary target of their toxic effects. Viroporins are short proteins (50 - 120 residues), have hydrophobic stretches of about 20 amino acids (amphipathic helix) involved in the integral membrane status of the protein, oligomerize (> tetramers) to form a hydrophilic pore and increase leucine and isoleucine but decrease glycine concentration in cells (Carrasco, 1994).

1.7.2 Mechanisms of late membrane permeabilization

As NS3 is not involved in cell infection that is the major cause of early membrane permeabilization, only late membrane permeabilization will be discussed briefly.

There are three major mechanisms of late membrane permeabilization. Firstly it can be a result of the decrease in membrane potential by an increase in Na^+ concentration and decrease in K^+ concentration. Progressive membrane damage induces a collapse of ionic gradients and disrupts membrane potential in virus-infected cells (Carrasco, 1994). Cell morphology at this time of infection usually appears normal under the phase-contrast microscope. Cell rounding and shrinkage takes place later when the membrane is highly damaged (Lopez-Rivas *et al.*, 1987).

Virus gene expression can also alter the pH and increase Ca^{2+} levels of infected cells. Membranes can also be permeabilized to hydrophilic antibiotics and low molecular weight compounds (Carrasco, 1994).

The third possibility is through the activation of phospholipase C to release choline from the membrane and form a high concentration of inositol-3-phosphate (IP3) with A2 archidonic acid released into the medium (Carrasco, 1994). Two consequences may be predicted if these two phospholipases are activated during the course of virus infection, firstly, the formation of prostaglandins and secondly the formation of diacylglycerol and lysophosphatidylcholine. The presence of these phospholipid moieties in the plasma membrane destabilizes the lipid bilayer and enhances membrane permeability.

Many examples can be found were small viral membrane proteins are implicated in cytotoxicty that will be briefly discussed in Chapter 4.

1.8 Aims

AHSV NS3 is a small nonstructural membrane associated protein that is likely to play a central role in the dynamics of virus dissemination in both vector (*Culicoides*) and host mammalian cells (usually equids). NS3 has been shown to be cytotoxic to Sf9 insect cells when expressed from a recombinant baculovirus (Stoltz, 1996; Van Staden *et al.*, 1998 & Smit, 1999). This effect is associated with disruption of the outer cellular membrane (Smit, 1999). The high level of AHSV NS3 variation can further influence its interaction with cellular components and efficacy of virus exit from infected cells. These findings appear to indicate that NS3 is one of the AHSV proteins that contribute to virus phenotypic characteristics that are thought to play a role in virulence.

The primary objective of this study was to investigate if NS3 of AHSV affects or contributes to events that possibly contribute to determining the virulence phenotype of AHSV. This was addressed by the following specific secondary aims:

1. To determine the role of the hydrophobic domains of NS3 in membrane interaction and cytotoxicty.

2. To establish the level of sequence variation of the S10 gene and NS3 protein within the AHSV serogroup and compare this to NS3 variation found in the NS3 of selected S.A. BTV isolates. It will be investigated if NS3 sequence data can be used as virulence and epidemiological markers.

3. To investigate the extent to which NS3 variation may influence or contribute to virus phenotypic properties such as membrane permeabilization and virus release that may be involved in virulence characteristics of different AHSV serotypes.

CHAPTER 2

MEMBRANE ASSOCIATION OF AHSV NONSTRUCTURAL PROTEIN NS3 DETERMINES ITS CYTOTOXICITY

2.1 INTRODUCTION

A number of highly conserved characteristics have been identified amongst all AHSV NS3 proteins (Van Staden *et al.*, 1995). These include three features of the N-terminal region of NS3, i.e. the presence of a second in-phase methionine codon for initiation of NS3A, a cluster of a least five proline residues between residues 22 to 34 and a stretch of highly conserved amino acids (78% identity and 96% similarity) from residues 46 to 90 (Fig 2.1). In addition, all NS3 proteins have two hydrophobic domains (HD) (residues 116 to 137 and 154 to 170) predicted to form transmembrane helices. These structural features are common to other orbivirus NS3 proteins, including that of BTV. The highly conserved nature of these domains motivated a long term study that aimed to elucidate their functional significance. This investigation focused on the prominent membrane association characteristics of NS3.

Stoltz (1994) and Smit (1999) constructed five NS3 mutants targeting the conserved domains to investigate their effect when expressed in host cells. These proteins, expressed as recombinant baculoviruses, displayed different cytotoxic profiles when expressed in Sf9 cells (Stoltz *et al.*, 1996; Smit, 1999; Van Staden *et al.*, 1998). The mutated first methionine start codon, conserved region and proline rich region NS3 proteins expressed from recombinant baculoviruses termed BAC-NS3A, BAC-Pro and BAC-CR (Smit, 1999) were all functionally similar to the wild-type NS3 protein in terms of cytotoxicty (Van Staden *et al.*, 1998; Smit, 1999) and were therefor considered as cytotoxic as wild-type NS3. However, the two mutant NS3 proteins, HD1 and HD2, expressed from BAC-HD1 and BAC-HD2, with substitutions in the first and second hydrophobic domains of NS3 respectively, differed significantly from wild-type NS3 in terms of their cytotoxic effects (Smit, 1999). Unlike wild-type NS3, expression of either HD1 or HD2 did not increase the permeability of the plasma membrane to trypan blue. Microscopic observation of infected cells clearly showed these cells to have intact plasma membranes (Smit, 1999). It therefore seemed that modification of either of the two predicted transmembrane regions of NS3 abolished or significantly reduced the cytotoxic effect of the expressed proteins, and that this was due to the fact that the mutant proteins were incapable of disrupting plasma membrane integrity. In an attempt to explain the reason for the apparent abrogated

cytotoxicity of the NS3 HD mutants it was investigated whether these mutants were able to localize to and/or associate with cellular membranes.

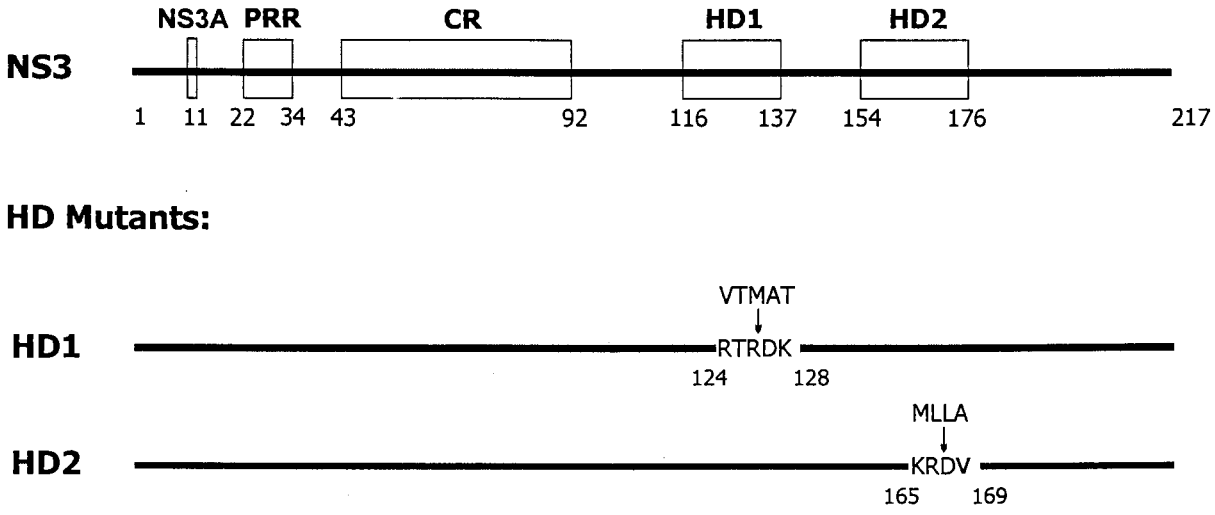


Fig 2.1 Schematic representation of the AHSV-3 NS3 protein. The positions of the proline rich region (PRR), highly conserved region (CR), the two hydrophobic domains (HD1 and HD2) and the NS3A initiation codon on NS3 are blocked and the corresponding amino acids positions are shown. The specific amino acid changes for the HD mutant constructs (Stoltz, 1994; Smit, 1999) are indicated.

2.2 MATERIALS AND METHODS

2.2.1 Cells and viruses

The AHSV-3 NS3 and the HD mutant proteins were previously constructed and expressed in the BAC-TO-BAC™ baculovirus expression system in Sf9 insect cells (Stoltz, 1994; Smit, 1999). The recombinant baculoviruses termed BAC-HD1 and BAC-HD2 expressed the NS3 HD mutant proteins directed towards the first and second HD respectively. Five non polar amino acids in HD1 (positions 124-128) and four non polar amino acids in HD2 (positions 165-169) were substituted with polar amino acids (Fig 2.1). These substitutions abolished the computer predicted membrane spanning potential of the HDs.

2.2.2 Pre-absorption of anti-AHSV-3 NS3 serum

Anti-β-galactosidase-AHSV-3 NS3 serum (Van Staden, 1993) was pre-absorbed prior to the immunofluorescence assays. Briefly Sf9 cells, grown in suspension, were collected by centifugation at 2000 x g for 10 min. The cell pellet was resuspended in fresh 1 x blocking buffer (1% w/v fat free milk powder in 1 x PBS) and the cells were lysed by passing the suspension through a 22 G needle several times. The anti-β-galactosidase-AHSV-3 NS3 serum was added to the lysed cell suspension to obtain a final dilution factor of 1:100. The cell suspension was incubated at room temperature with gentle agitation for 2 h. The cell debri was removed by centrifugation as described above. Supernatant containing unbound antibodies was transferred to a sterile tube and kept at –20°C for use in the immunofluorescence assays.

2.2.3 Immunofluorescence assays

Sterile coverslips were prepared by submerging them in 70% ethanol, briefly drawn through a flame and placed onto the bottom of a well in a 6-well tissue culture plate. Sf9 cells were seeded at a density of 1 x 10^6 cells into the wells and left to attach for 1 h. The seeding medium was removed and replaced with 1 ml of supplemented Graces insect medium (Highveld Biological) to which the virus inoculum was added to obtain an MOI of 5. The inoculum was removed an hour later and growth medium replaced as described above. The infected cells were incubated at 27°C until used. Coverslips were removed 27 h p.i., rinsed in 1 x PBS and incubated for 30 min in blocking solution (5% w/v fat-free milk powder in 1 x PBS). Coverslips were then incubated with pre-absorbed anti-β-galactosidase-AHSV-3 NS3 serum (diluted 1:100) for 1 h at room temperature and washed 3 times in wash buffer (0.5% v/v Tween-20 in PBS) for 5 min periods. Bound primary antibody was detected after a 45 min incubation with fluorescein-conjugated goat anti-rabbit IgG (Sigma) (diluted 1:250), washed as above and mounted onto glass slides in glycerol-PBS (90:10 v/v). Cells were examined under a Zeiss fluorescent microscope. For fixed (permeabilized) cell analyzes, cells were rinsed in 1 x PBS, fixed for 30 sec with methanol-acetone (50:50 v/v) and followed by the same blocking and immunolabeling procedures as above.

2.2.4 *In vitro* transcriptions

The AHSV-3 S10 gene and mutated HD versions had been cloned into the *Bam HI* site of pBS (Stoltz, 1994; Smit, 1999). To determine the orientation of the cloned genes, approximately 1 µg of the recombinant plasmids were digested with 10 U *Eco RI* (that digests in the multiple cloning site) and 10 U of *Bgl II* (a restriction enzyme that has a site in the S10 gene but not pBS) in 1 x restriction buffer H (all Roche Diagnostics) for 2 h at 37°C. The digestions were analyzed on a 1% agarose gel stained with 3.5 µl ethidium bromide (10mg/ml) that had been electrophoresed for 30 min at 80 V.

mRNA transcripts of S10 and the HD mutated genes were synthesized from the respective pBS constructs under the control of the T7 polymerase promoter. Recombinant plasmids (10 µg) were digested with 1 U *Hind III* in buffer M (Roche Diagnostics) for 2 h at 37°C to linearize the genes at the 3' end. A small amount of the digestion reaction was electrophoresed on a 0.8% agarose gel (as described above) to verify that the plasmids were completely linearized. Linearized plasmids were purified with phenol/chloroform extractions and ethanol precipitations (Sambrook et al., 1989). Transcription reactions contained 6 µl of linearized plasmids (250 ng/µl), 1 µl Rnase Inhibitor (Amersham), 2 µl 2 x transcription buffer, 2.5 mM rNTPs and 1 µl T7 RNA polymerase (Roche Diagnostics). Reactions were incubated at 37°C for 1 h and transcription efficacy was evaluated after electrophoresis on a 0.8% agarose gel. All gel chambers and buffers used for RNA agarose gels were prepared with DEPC treated UHQ.

2.2.5 *In vitro* translations

mRNA transcripts were *in vitro* translated using the rabbit reticulocyte lysate (RRL) system (Amersham) in the presence or absence of canine pancreatic microsomal membranes (CMM) (Roche Diagnostics) according to manufacturer's procedures. The reactions consisted of 4 µl 12.5 x translation mix (without methionine), 2 µl 2.5M potassium acetate, 1 µl 25 mM magnesiun acetate, 4 µl [^{35}S]-methionine (10 µCi/µl, Separations), 1 µg RNA sample and 20 µl RRL in a total volume of 50 µl made up with Rnase-free water. Reactions were scaled down to 25 µl reaction volumes with equal success. Translations performed in the presence of CMM included 1 µl of the CMM extract. The protein samples were diluted 1:10 with 2 x PSB, boiled for 4 min and analysing by SDS-PAGE. After electrophoresis (120 V for 1 h 45 min) the gels were fixed in 7% acetic acid for 30 min at room temperature, rinsed in water and placed onto Whatman 3MM filter paper, covered with plastic cling wrap and dried for 1 h at 60°C. Dried gels were exposed to autoradiography film overnight and the films were developed using standard procedures (Sambrook *et al.*, 1989).

2.2.6 Membrane association assays

Membrane association of NS3, HD1 and HD2 proteins was analyzed by treating 5µl aliquots of the *in vitro* translated samples in the presence of microsomal membranes with either 100µl (a) 0.1M $NaCO_3$, pH 11.5, that converts closed vesicles into open sheets (Fujiki *et al.,* 1982), (b) solubilization buffer (10mM Tris.HCl pH 7.5, 10mM KCl, 5mM $MgCl_2$, 1% NP40) that solubilizes membrane proteins, or (c) 0.5M NaCl that solubilizes peripheral membrane proteins. The reactions were incubated on ice for 30

min. The particulate and supernatant fractions were separated by centrifugation at 4°C for 1 h at 18 000 x g in a Sigma 2K15 centrifuge and the resultant particulate fraction resuspended in 100µl of 1 x PBS. Protein samples were resolved by 12% SDS-PAGE, fixed for 1 h in 7% acetic acid, dried and autoradiographed. As a control the NS3, HD1 and HD2 proteins were *in vitro* translated in the absence of microsomal membranes, treated with the carbonate buffer and resolved as described.

Membrane association characteristics of NS3, HD1 and HD2 with CMM were further examined by proteinase K digestions (Echeverri & Dasgupta, 1995). Aliqouts of 8 µl of the *in vitro* translated samples were digested with 200 µg/ml proteinase K with or without 1% NP40 for 1 h at 4°C. Reactions were stopped by addition of 8 mM phenylmethan sulfonylfluorid (Merck) and incubated for an additional 10 min on ice. Protein samples were analyzed by SDS-PAGE and autoradiography as previously described.

2.3 RESULTS

The experiments described in this chapter are mainly focused on trying to provide an explanation of why mutations in the hydrophobic domains of NS3 of AHSV resulted in a loss of NS3 associated cytotoxicity in the Sf9 cells in which NS3 and the HD mutants were expressed.

2.3.1 Plasma membrane localization of NS3 and hydrophobic domain mutants

To investigate whether the localization of HD1 or HD2 to the cell surface was impaired, infected cells were analyzed by indirect immunofluorescence (Fig 2.2). Cells were infected with recombinants BAC-NS3, BAC-HD1, BAC-HD2 or a baculovirus recombinant expressing AHSV NS2 (control). Intracellular NS3 is inaccessible to serum when cell membranes are still intact (up to 27 h p.i.; Smit, 1999) and have not been permeabilized by fixation. Unfixed cells expressing NS3 clearly showed a homogenous fluorescence of the entire cell surface (Fig 2.2B). In contrast, cells expressing HD1 (Fig 2.2C) and HD2 (Fig 2.2D) only gave a low background signal, similar to control cells (Fig 2.2A), indicating the absence of NS3-related proteins or accessible epitopes on the surface of these cells. Fixed and permeabilized cells displayed a bright perinuclear cytoplasmic staining for all three recombinants (not shown), confirming protein expression in all cases.

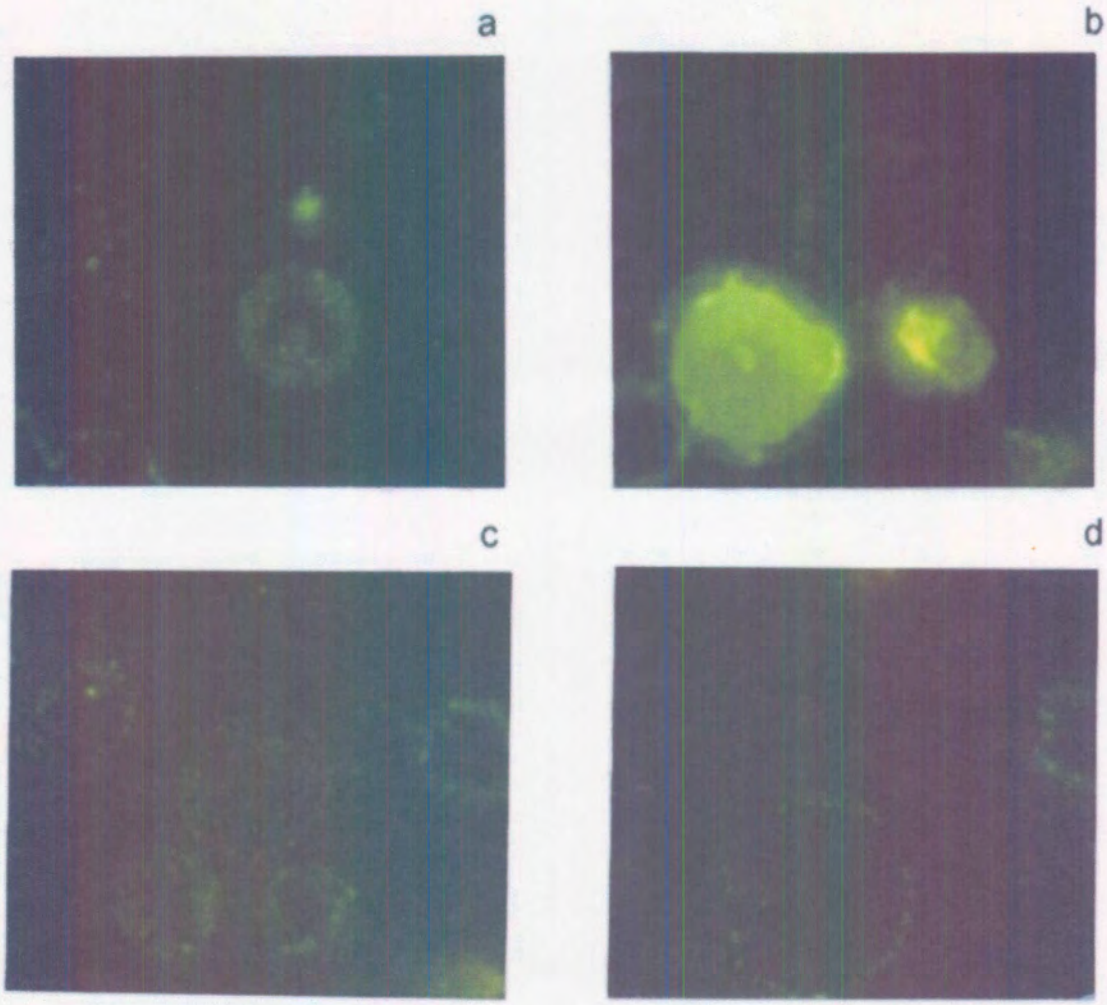


Fig 2.2 Localization of NS3 and HD mutants to the cytoplasmic membrane. Sf9 cells expressing AHSV NS2 as a control (a), BAC-NS3 (b), BAC-HD1 (c) and BAC-HD2 (d) were analyzed by indirect immunofluoresence at 27 h p.i. Unfixed cells were blocked and reacted with anti-β-galactosidase-AHSV-2 NS3 serum followed by a fluorescent conjugate. Magnification 100x.

2.3.2 Membrane association potential of NS3 and HD mutants

As the two mutant proteins were not localized to the outer plasma membrane, their possible association with membrane structures was investigated and compared to that of NS3. This investigation used an artificial mini-cell system. mRNA transcripts of the genes were synthesized from copies of the recombinant pBS clones (Fig 2.3A). The multiple cloning site (MCS) of pBS is flanked by the T7 and T3 RNA polymerase promoters on opposite sides to facilitate transcription irrespective of gene orientation in the vector. To establish which promoter to use, the orientation of the S10 gene was determined by double digests with *Eco RI* (that cuts in the MCS) and *Bgl II* (that cuts in S10 about 500 bp from the 5' end). Gene inserts in the T7 orientation would excise a fragment of 500 bp while inserts in the T3 orientation would yield a 280 bp fragment. Results of the double digestion indicated the presence of a DNA band of 500 bp and all three of the cloned genes were therefor under the control of the T7 promoter (results not shown).

As a control for the *in vitro* transcription reactions, the encoding AHSV VP6 gene, S9, (gift from P. de Waal,) was used (Fig 2.3A, lane a) that gave a mRNA transcript predicted to be 1169 nucleotides in length. The S10 gene *in vitro* transcribed a transcript of about 780 nucleotides (Fig 2.3A, lane b and c) being smaller than that of the VP6 gene. The yield of the *in vitro* transcribed S10 gene products were similar (Fig 2.3B) for NS3 (lane a), HD1 (lane b) and HD2 (lane c). The upper bands on the agarose gel were the linearized recombinant pBS templates from which the mRNA was synthesized. The mRNA transcripts were directly translated with the RRL system (Amersham) (Fig 2.4). Prominent radio signals were observed in the size range expected for AHSV NS3 (about 24 K) indicating the presence of AHSV NS3 (lane b), HD1 (lane c) and HD2 (lane d). The positive control of the kit was included (lane a) while the negative control contained no exogenously added RNA (lane e).

In vitro translations were then conducted in the presence of canine microsomal membranes, fractionated in different buffers (see 2.2.6) and analyzed by SDS-PAGE and autoradiography. The results obtained are shown in Fig. 2.5. Sodium carbonate at alkaline pH precipitates membranes and retains integral membrane proteins, while releasing cytosolic proteins (Fujiki *et al.,* 1982; Tiganos *et al.,* 1998). Therefor proteins localized to the particulate fraction of the carbonate treatment

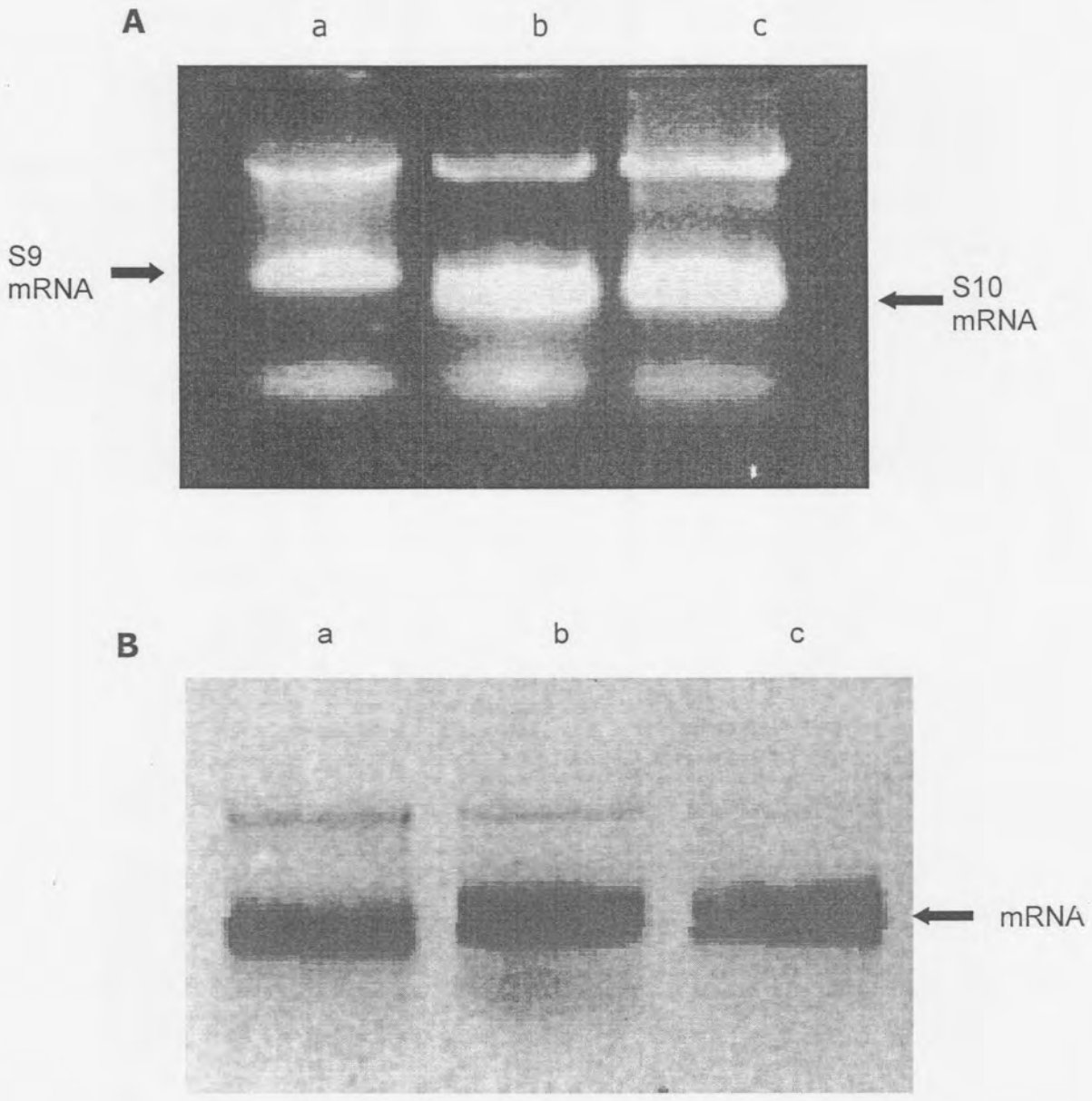


Fig 2.3 Synthesis of NS3 and HD gene mRNA transcripts. (A) *In vitro* transcripts were synthesized from linearized pBS under the control of the T7 promoter and analyzed on a 0.8% agarose gel stained with ethidium bromide. S9 mRNA transcript (1169 nt) served as a size control (a), S10 mRNA transcripts were present as smaller products (b and c). (B) Yield of mRNA from transcription of the S10 gene (a), HD1 (b) and HD2 (c) mutated genes.

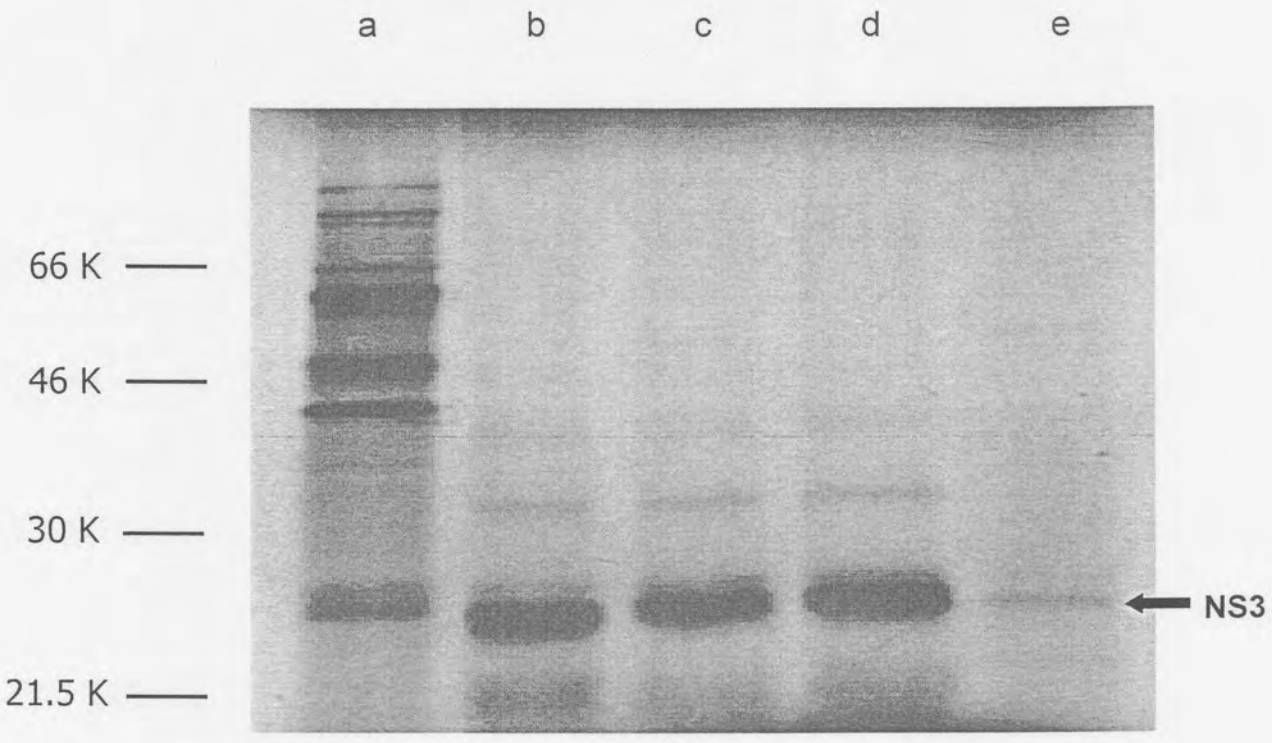


Fig 2.4 *In vitro* translation of mRNA using the RRL system. Protein samples were analyzed by 12% SDS-PAGE and autoradiography. Radiolabeled NS3 (b), HD1 (c) and HD2 (d) proteins were synthesized from mRNA transcripts. A control RNA sample supplied with the kit was included (a) and the sizes of the protein rainbow marker are as indicated. As a negative control a sample containing no exogenously added RNA was translated (e).

would indicate that they are integral membrane proteins. Wild type NS3 was present in the supernatant (lane a) and in the particulate fraction (lane b) after treatment with 0.1 M $NaCO_3$ (pH 11.5) while the two mutant proteins HD1 and HD2 were found exclusively within the supernatant fractions. Treatment with solubilization buffer would release proteins anchored in microsomal membranes into the supernatant fraction of the samples. Wild type NS3 and the HD mutants were only found in the supernatant fraction (lane c) after solubilization treatment. The high salt buffer releases membrane associated but not integral membrane proteins from the particulate microsome fraction. It would be expected that peripheral membrane proteins would be solubilized in a high salt environment and thus be found in the supernatant fraction of the 0.5 M NaCl treatment. Wild-type NS3 was located to both supernatant (lane e) and particulate (lane f) fractions following 0.5 M NaCl treatment. The HD1 and HD2 mutants were however only present in the supernatant fraction under the same conditions. Therefore NS3 was localized in the membrane fractions of the treated microsomes, probably as an integral and not a peripheral membrane protein. The results of the HD1 and HD2 mutants indicate that neither of the two mutant proteins was associated with the microsomes as integral or as peripheral membrane proteins. The presence of NS3 in the supernatant fractions of the carbonate treatments can be attributed to its inherent ability to permeabilize and destabilize membranes, probably causing microsome disruption and fragmentation. As expected, all NS3 was present in the supernatant fraction after solubilization, confirming its membrane association.

To investigate whether the proteins were targeted to the canine microsomal membranes but not inserted into the membranes proteinase K digestions were done as described (see 2.2.6). Autoradiographs of the proteins samples following proteinase K digestions (Fig 2.6) showed that NS3 (lane a), HD1 (lane d) and HD2 (lane g) were completely digested by proteinase K in the presence of NP40 that would solubilze membranes and release bound proteins. Omission of NP40 from the reactions resulted in the degradation of NS3 (lane b) while the HD mutants remained intact under the same conditions (lane e & h). A clear difference between HD1 (lane e) and HD2 (lane h) was the remaining concentration of the undigested protein samples. Only a portion of HD1 was undigested (lane e) in comparison to the control sample that indicated the total protein concentration used in these

digestions (lane f). The majority of HD2 (lane h) was protected in comparison to the equivalent undigested control sample (lane i)

From these results it is clear that mutations introduced into either of the two hydrophobic regions affected the ability of NS3 to interact with membranes in a stable conformation. This would explain the absence of HD1 and HD2 from the cell surface and the diminished cytotoxic effect of these two mutant proteins.

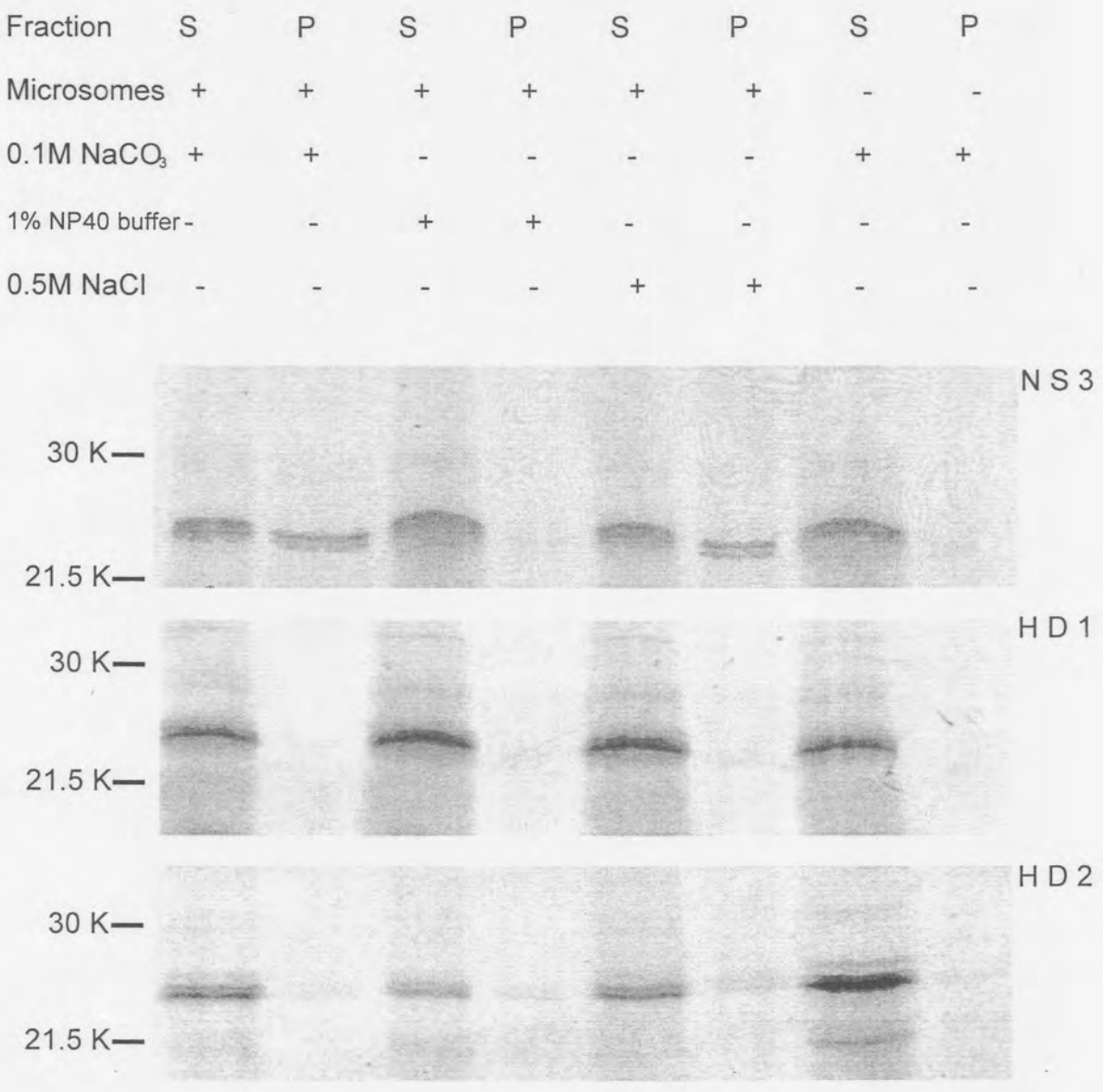


Fig 2.5 Membrane association of NS3, HD1 and HD2. Full length NS3 mRNA or mRNA of the hydrophobic domain mutant constructs HD1 and HD2 were *in vitro* translated in the presence of microsomal membranes and treated with either 0.1M $NaCO_3$ pH 11.5, solubilization buffer containing 1% NP40, or 0.5M NaCl as indicated and separated into supernatant (S) and particulate (P) fractions by centrifugation. *In vitro* translations in the absence of microsomal membranes followed by 0.1M $NaCO_3$ treatment served as a control. Sizes of molecular weight markers are as indicated.

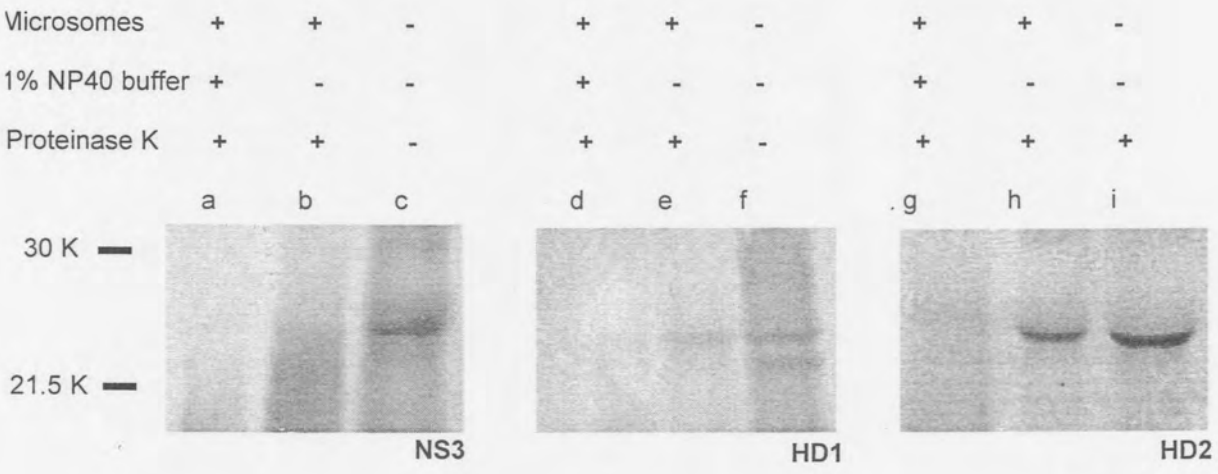


Fig 2.6 Membrane targeting of NS3, HD1 and HD2. The proteins were synthesized by *in vitro* translation reactions and CMMs as described and digested with proteinase K in the absence or presence of 1% NP40. The protein samples were analyzed by 12% SDS-PAGE and autoradiography. Undigested control protein samples for NS3 (c), HD1 (f) and HD2 (i) indicated the concentration of the samples used for the assay. Sizes of the molecular weight markers are as indicated.

2.4 DISCUSSION

Expression of AHSV nonstructural protein NS3 leads to subsequent death of infected host cells (Van Staden *et al.*, 1995), probably due to an interaction with cellular membrane components (Stoltz *et al.,* 1996). The aim of this study was to investigate the roles of the hydrophobic domains in this membrane interaction. This was conducted through establishing the reason for the abrogated cytotoxicity of the HD NS3 mutants by analyzing their membrane localization and association abilities in comparison to the wild type NS3.

Previous investigations indicated that both hydrophobic domains of BTV NS3 span the membrane (Bansal *et al.,* 1998). BTV NS3 was also shown to be glycosylated (Wu *et al.,* 1992) and it has been suggested that glycosylation may prevent BTV NS3 from degradation (Bansal *et al.,* 1998). Despite certain conserved features, AHSV NS3 differs significantly from BTV NS3. There is no evidence that glycosylation of AHSV NS3 plays an important role as NS3 of certain serotypes have no predicted glycosylation sequence motifs. BTV NS3 shows high levels of expression in the baculovirus system (French *et al.*, 1989), while AHSV NS3 is expressed in lower levels and is only detected by immunoblots or radiolabeling (Van Staden and Huismans, 1991). Further, the high mortality rate of AHS clearly indicates that it is a more severe disease than that of bluetongue. These differences justify a separate investigation to clarify the AHSV NS3 structure/function relationship, and specifically its role in viral morphogenesis and pathogenesis.

The observed differences between NS3 and the mutants HD1 and HD2, in which the first and second transmembrane domains were mutated respectively, could be ascribed to a variety of different factors including the inability of HD1 and HD2 proteins to interact with membranes. It is furthermore possible that an abnormal configuration of NS3 mutants and loss of functional domains on the proteins could affect their transport and stability. To monitor the localization of NS3 and the mutant proteins HD1 and HD2 to the plasma membrane, infected cells were subjected to indirect immunofluorescence studies. Whereas NS3 was present on the surface of unfixed cells, no HD1 or HD2 was detected under the same conditions. Labeling of fixed cells, however, indicated similar levels of intracellular expression for all three proteins. The mutant NS3 proteins HD1 and HD2 are

therefor either retained along the biosynthetic protein transport system and fail to reach the plasma membrane, or are not integrated into the intracytoplasmic membrane components of the cell in a stable configuration. Subcellular targeting of viral (and cellular) membrane proteins is not solely determined by the sequences of the transmembrane regions. Retention sequences or transport sequences in the luminal and/or cytoplasmic domains of the protein including glycosylation, myristylation or multimerization may further influence targeting (see Chapter 1 for more detail). Specific mechanisms operate to recognize and retain incorrectly folded proteins in the ER preventing any further processing (Doms *et al.,* 1993; Hegde & Lingappa, 1997).

The reason for the impaired surface membrane localization ability of both the hydrophobic domain mutants HD1 and HD2 was investigated. While NS3 was shown to be an integral membrane protein, HD1 and HD2 were not anchored in the microsomal membranes. Disruption of one of the two hydrophobic domains of NS3 affected the stable membrane interaction in an *in vitro* system. This is likely to be associated with the membrane topology of NS3. Van Staden *et al*, (1995) previously modeled a computer-based topology for AHSV NS3 with both hydrophobic domains spanning the cell membrane and the N and C termini exposed cytoplasmically (Van Staden *et al.,* 1995). This model was substantiated experimentally for BTV NS3, where mutant proteins with deletions in either of the two hydrophobic domains were still processed to the Golgi complex (Bansal *et al.,* 1998), i.e. one intact hydrophobic domain was sufficient to confer membrane targeting and processing from the ER to the Golgi complex. It is not clear from this report whether the mutant BTV proteins were further transported to the cell surface. An alternative model was proposed for EHDV NS3 (Jensen & Wilson, 1995), in which the first hydrophobic domain may function as an insertion signal peptide that is translocated into the ER lumen and lies buried in the lipid bilayer surface. The second hydrophobic domain functions as a transmembrane segment which then anchors NS3 as an integral membrane protein. Our data does not exclude one of the two models. It is however evident that only a very specific membrane associated conformation of AHSV NS3, dependant on the structure of both hydrophobic domains, makes it cytotoxic to host cells.

In order to determine whether the apparent lack of association of the HD mutants with the CMMs was a result of the proteins being targeted and internalized rather

than anchored into the membranes, proteinase K digestions were done with the *in vitro* translated protein samples (see 2.2.6). NS3, that served as an inserted protein control, showed incomplete degradation in the presence of proteinase K but absence of NP40 (Fig 2.6, lane b). This could be attributed to a possible permeabilization effect of NS3 on the CMMs. The presence of HD1 and HD2 (Fig 2.6, lanes e and h respectively) indicated that the mutants were afforded a certain degree of protection from proteinase K digestions. This indicated that the proteins were targeted to, but not anchored into the membranes as a consequence of the disrupted hydrophobic domains. The enhanced protection afforded to HD2 in comparison to HD1 is unclear and requires further investigation. This difference may be indicative of a stronger membrane targeting signal present in HD2 as opposed to that of HD1.

These experiments did not address the precise mechanism whereby NS3 disrupts the cellular membrane and increases membrane permeability, although it was shown to be dependent on its membrane-associated conformation. The function of NS3 in the viral life cycle could therefore also include a role in controlling the biochemical homeostasis of infected cells through regulating membrane permeability. NS3 may therefore play a key role in both the viral pathogenesis and viral release processes that regulate the disease progression of African horsesickness.

The definite association between AHSV NS3 and cytotoxicity indicated a possibility of identifying virulence markers in AHSV NS3. In order to investigate this question it was necessary to study the level of variation of AHSV NS3 and more specifically that of the HDs between avirulent and virulent strains.

CHAPTER 3

VARIATION OF AHSV AND BTV NS3 IN SOUTHERN AFRICA

3.1 INTRODUCTION

The current vaccination strategy in S.A. is based on the use of a combination of live attenuated strains representing the different AHSV serotypes (Coetzer & Erasmus, 1994). These live vaccines have been attenuated by serial cell culture passages. The size of AHSV plaques in cell culture were, in the past, found to be useful virulence markers of AHSV. Small plaques were associated with virulent viruses while larger plaques were associated with less virulent viruses and these were subsequently attenuated to produce AHSV vaccine strains (Coetzer & Erasmus, 1994). However, the use of plaque size is not a reliable indication of virulence and this approach has lost favour (P. Howell, personal communication). The use of a molecular marker for virulence has not been explored. A new and definitive marker of the virulence of AHSV is therefor in demand. In view of the possible association of NS3 with virulence (O' Hara *et al.*, 1998; see Chapters 2 and 4) we investigated if NS3 sequence could in any way be used as a marker for AHSV virulence. To establish the value of NS3 as a virulence marker we studied the NS3 sequence variation level between several recent virulent field isolates, reference strains and attenuated vaccine strains of southern African AHSVs.

Molecular phylogeny is the study of evolutionary relationships among organisms using molecular biology techniques. The rapid accumulation of DNA sequences over the last 20 years has had a great impact on molecular phylogeny. Phylogenetic programmes are also constantly updated to accommodate larger volumes and more complex sequence data. Over and above studying the evolutionary relationships between organisms, phylogenetic studies open a door into the field of molecular epidemiology enabling one to track the origins, extent and spread of an epidemic outbreak. This has been of particular interest in the field of virology and has been used for rabies virus, influenza virus, HIV and dengue virus to mention but a few.

AHSV S10 gene sequences are available for all nine AHSV serotypes and S10 is the most common AHSV gene sequenced. Many of the parental virus strains for which these sequences have been determined originated from South Africa or the Middle East. Little detail is available regarding the date and location of isolation, number of times passaged in cell culture or organ and species of isolation for most of these strains. This limits the use of the sequence data to phylogeny. These AHSV S10 gene and NS3 protein phylogenetic studies have described three distinct NS3 genetic lineages designated groups α, β and γ respectively with a maximum variation of 35% between different serotypes (Sailleau *et al.*, 1997; Martin *et al.*, 1998). Phylogeny in combination with the relevant field data has become greatly beneficial in the field of viral epidemiology. In concurrence with serotyping it may be possible to use NS3 sequence data as an epidemiological marker to distinguish between different outbreaks of the same serotype as well as between field isolates and live attenuated vaccine strains.

BTV S10 gene and encoding NS3 protein sequences have been more extensively investigated than those of AHSV. BTV NS3 groups into three monophyletic clusters (Bonneau *et al.*, 1999) independent of BTV serotype, year of isolation and host species of isolation (Pierce *et al.*, 1998). One of the monophyletic clusters consists of BTV isolates from China and an Australian isolate indicating a possible segregation based on geographic location (Bonneau *et al.*, 1999). This suggests that the viruses in the different clusters were subjected to different evolutionary pathways leading to diversification. The variation within the NS3 protein of BTV isolates is reported to be only 7% (Hwang *et al.*, 1992*a*). Recent international studies on BTV S10 have focused on strains circulating in the U.S.A., China and Australia and limited sequence data is available for S10 gene and encoding NS3 protein of S.A. BTV isolates. It was therefor necessary to compare the level of sequence variation found amongst S10 and NS3 of S.A. BTVs to the international reports on BTV S10 and NS3 variation as well as to the level of AHSV S10 and NS3 variation. This study would also contribute to the global epidemiological investigation of BTV. Several historical isolates dating to the early 1900s were part of this investigation that

additionally enabled the variation in BTV S10 to be monitored over a time period of *c.a.* 100 years in S.A.

To investigate these questions the S10 sequences of 19 AHSV isolates and 20 S.A. BTV isolates were determined by direct RT-PCR sequencing and the sequence data was compared and used for further phylogenetic studies.

3.2 MATERIALS AND METHODS

3.2.1. Cells and viruses

Monolayer cultures of Vero cells were propagated in minimal essential medium (Highveld Biological) supplemented with 5% fetal calf serum (FCS) (Highveld Biological), penicillin (0.12 mg/ml), streptomycin (0.12 mg/ml) and Fungizone (0.3 μg/ml, all Highveld Biological). Cells were infected with AHSV or BTV isolates at a confluency of 80%. The OIE AHS Reference Laboratory at the Onderstepoort Veterinary Institute (OVI), South Africa provided and serotyped all the viruses.

Table 3.1 List of AHSV isolates used in this study.

Serotype	Virus	Virus details	Geographic location	Year of isolation	Accession number
2	S2FLD	AHSV-2 FIELD	UN	UN	AF276700
2	S2VAC	S2 Vaccine-125*	UN	UN	AF276693
2	S2REF	S2REF-82/61	UN	1961	AF276694
3	S3FLD1	M322/97†	Gauteng	1997	AF276695
3	S3FLD2	HS14/98	Kwa-Zulu Natal	1998	AF276699
3	S3VAC	S3 Vaccine	UN	UN	AF276701
3	S3REF	S3 Laboratory strain	UN	UN	D12479
4	S4FLD	HS39/97	Namibia	1997	AF276696
5	S5REF	S5 Laboratory strain	UN	UN	U60188
6	S6FLD1	HS33-4/98	Free State	1998	AF276688
6	S6FLD2	HS6/98	Kwa-Zulu Natal	1998	AF276687
6	S6VAC	S6 Vaccine	UN	UN	AF276686
6	S6REF	S6 Laboratory strain	UN	UN	AF276689
7	S7FLD1	HS43/98	Gauteng	1998	AF276697
7	S7FLD2	HS45/978	Gauteng	1998	AF276698
7	S7VAC	S7 Vaccine	UN	UN	AF276701
8	S8FLD1	HS2/98	Gauteng	1998	AF276692
8	S8FLD2	HS7/98	UN	1998	AF276691
8	S8VAC	S8 Vaccine	UN	UN	AF276690
8	S8REF	S8 Laboratory strain	UN	UN	AF276658
9	S9REF	S9 Laboratory strain	UN	UN	D12480

*S2VAC was produced from the AHSV-2 reference strain 82/61 that was isolated in 1961. †M322/97 was isolated from the spleen of a dead dog. All the other field isolates were isolated from spleen or lung samples of dead horses. FLD denotes field strain. UN denotes that the information is unknown. VAC denotes vaccine strain. REF denotes an Onderstepoort reference laboratory strain.

The AHSV strains that were used in this study included recent field isolates of serotypes 2, 3, 4, 6, 7 and 8 obtained from fatal AHSV infections of horses as well as a dog and current vaccine and laboratory reference strains (Table 3.1). Bluetongue virus isolates (serotypes 1, 2, 3, 4, 8, 11 and 18) received from the OVI are given in Table 3.2.

Table 3.2 South African BTV isolates received from the OVI used in this study.

Serotype	Virus	Virus Details	Year of isolation
1	S1VAC	5012 Vaccine	1958
1	S1REF	Reference strain	1958
1	S1F1	S1 Field	1999
1	S1F2	S2 Field	1999
2	S2REF	Reference strain	1958
2	S2F	Ramsen Field	1999
3	S3VAC	8231 Vaccine	1944
3	S3REF	Reference strain	1944
3	S3F	G717 Field	1999
4	S4VAC	79043 Vaccine	1900
4	S4REF	Reference strain	1900
4	S4F1	G712 Field	1999
4	S4F2	P524 Field	1999
8	S8VAC	8438 Vaccine	1937
8	S8REF	Reference strain	1937
8	S8F	GA05 Field	1999
11	S11VAC	4578 Vaccine	1944
11	S11REF	Reference strain	1944
11	S11F	G704 Field	1999
18	S18F	B1 Field	1999
18	S18REF	Reference Strain	UN

F denotes field strains. VAC denotes vaccine strains. REF denotes reference strains. UN is information unknown.

3.2.2 RNA isolations, complementary DNA synthesis and PCR

Total RNA was extracted from two 75 cm^3 flasks of AHSV infected Vero cells showing 80 - 90% CPE, usually 3 to 5 days post infection, using TRIZOL™ reagent (Gibco BRL) according to manufacturers recommendations. The RNA sample was resuspended in diethylpyrocarbonate (DEPC) treated water and stored at -20°C. The dsRNA of S10 was reverse transcribed into a complementary DNA (cDNA) copy using primers that annealed to the 3' and 5' terminal regions of the RNA segment, namely NS3pEco (5' *cggaattcgtaagtcgttattccgg*) and NS3pBam (5' *cgggatccgtttaaattatcccttg*) respectively. The primers contained restriction enzyme sites (underlined) for cloning purposes. Complementary DNA was synthesized as previously described (Zientara *et al.*, 1998; Wade-Evans, 1990). Briefly, 250 – 500 ng of RNA was denatured with an equal volume of 10 mM methylmercuric hydroxide (MMOH) for 10 min at room temperature. The MMOH was reduced by addition of 2 µl 0.7 M β-mercaptoethanol in the

presence of 159 U RNase inhibitor (Amersham Life Sciences) and left for a further 5 min at room temperature. The denatured RNA was added to a cDNA reaction mix containing 100 pmol of each primer, 2 µl of 10 mM dNTP mix (in sodium salt), 2.4 µl 5x reaction buffer and 5 U AMV reverse transcriptase (Promega). The reaction was incubated for 90 min at 42°C. The S10 cDNA was amplified in a subsequent PCR using 1-4 µl of cDNA, 10x reaction buffer including optimized $MgCl_2$ concentration, 4 µl of each primer (100 ng/µl), 5 µl 1 mM dNTPs and 1 U Taq polymerase (Takara ExTaq) in a reaction volume of 50 µl. The reaction conditions were set for one cycle of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 45 sec at 50°C and 1 min at 72°C and a final cycle as above except that the elongation time was extended for a further 4 min.

The same strategy was used to amplify the BTV S10 genes except that BTV S10 specific primers were used. The S10 terminal primers, BTVF1 (5' *gttaaaaagtgtcg tgcc*) and BTVR1 (5' *gttagtgtgtagagccgcg*) annealed to the terminal regions of BTV S10 and were used to synthesize BTV S10 specific cDNA and in subsequent PCRs.

3.2.3 DNA sequencing and analysis

S10 PCR amplicons were purified using a commercial purification kit (Roche Diagnostics) and sequenced according to the manufacturer's recommendations using the ABI 377 automated sequencer (Perkin Elmer). In addition to the above-mentioned AHSV S10 primers, an additional internal primer, NS3C2.rev (5' *gcc cca ctc gca cca g*) was designed to sequence the 5' region of the gene. An additional BTV S10 primer BTVR2 (5' *gcg tac gat gcg aat gca gc*), designed to anneal to a conserved region within BTV S10, was used in BTV S10 sequencing reactions to obtain the full length BTV S10 ORF sequence. The S10 sequences were aligned and translated into NS3 amino acid sequences that were subsequently aligned using ClustalX (Thompson *et al.*, 1997). The aligned NS3 sequences were used to generate a table of mean pairwise distances (PAUP ver 4.0b5) to evaluate the variation within NS3. Pairwise distances between the aligned S10 sequences were calculated using the uncorrected 'p' parameter settings of PAUP. Accession numbers for the AHSV and BTV protein sequences retrieved from Genbank used in variation comparisons are listed in Table 3.3. The phylogeny of AHSV NS3 and BTV S10 was investigated with the construction of phylogenetic trees using the neighbour joining and parsimony methods (PAUP ver 4.0b5). The HKY 85 distance setting was used to construct the neighbour joining trees. A gamma test was done on the AHSV aligned S10 nucleotide sequences to evaluate whether the genes displayed unequal rates of mutation. Bootstrap analysis was done (1000 replicates) and confidence scores are shown on the branches of the neighbour joining and parsimony trees (PAUP ver 4.0b5).

Table 3.3 Accession numbers of additional sequences used in this study.

AHSV Proteins	Accession number
VP2	D26570, AF43926, AF021235, Z26316, U21956, U01832, M90697
VP3	AF021236, D26572, M94312
VP5	AF021237, D26571, U74489, M94731
VP6	U33000, U19881
VP7	AF21238, U90337, D12533, X56676, AF27209
NS1	D11390, U73658, U01069
Related Orbiviruses	
CHV (Palyam serogroup)	AB018091
EHDV	L29022, L29023
BTV	AF135227, AF135224, AF44707, AF13228, AF135229, AF044373, AF044378, AF044710
BRDV	M83197

3.2.4 *In vitro* translations and protein analysis

Purified dsRNA was obtained from the total TRIZOL™ RNA extractions by addition of LiCl to final concentration of 2 M and the ribosomal RNA was precipitated overnight at 4°C. The dsRNA was collected after removing the rRNA by centrifugation at 20 000 rpm for 15 min and precipitating the supernatant with 2.5 volumes of ice cold 96% ethanol and 0.2 M NaCl for 1 h at – 70°C followed by centrifugation for 10 min at 12 000 rpm. The dsRNA sample was dissolved in 50 µl DEPC treated UHQ per 75 cm^3 flask of infected cells.

The rabbit reticulolycate lysate (RRL) system (Amersham Life Sciences) was used for *in vitro* translation reactions with 1 µg of purified dsRNA. The translation reactions were scaled down to half reaction volumes that included 2 µl of the translation mix without methionine, 1 µl 2.5 M potasium acetate, 0.5 µl 25 mM magnesium acetate, 2 µl [^{35}S]-methionine and 10 µl rabbit reticulolycate. The reactions were incubated at 30°C for 90 min. *In vitro* translated protein samples were diluted 1 in 10 with 2 x PSB and analyzed using standard 15% SDS-PAGE on large 15 x 15 cm^2 gels and autoradiography.

3.3 RESULTS

3.3.1 AHSV NS3 sequence analysis

A total of 19 AHSV strains including recent field isolates and vaccine strains were obtained from the OIE Reference Laboratory at the OVI. The NS3 sequences of these S.A. AHSV strains were determined, aligned and analyzed to identify conserved and variable protein domains (Fig 3.1). The ORF of AHSV S10 encoded a protein of 217 amino acids in the case of AHSV-3, -4, -5, -6, -7, -8 and -9 and 218 amino acids in the case of AHSV-2. Conserved regions identified by Van Staden *et al.* (1995) were evident namely the NS3A initiation codon, a proline rich region, a highly conserved region from amino acids 43-92, and two predicted hydrophobic domains. Most of the amino acid differences grouped within three regions, namely the first 43 residues at the N-terminal, the region between residues 93 and 153 and the 15 C-terminal residues of NS3. The most variable region (82.4% variation) was located between amino acids 136 and 153. The NS3 membrane associated model proposed by Van Staden *et al.* (1995) maps this region to the exterior of the cell membrane. The NS3 proteins of all AHSV serotypes with the exception of serotype 2, showed a conserved cysteine residue in position 123. In AHSV-2 the cysteine is located in position 120 (Fig 3.1). The position of the second cysteine residue (164) is, however, fully conserved amongst all serotypes.

In addition to the above-mentioned domains, a highly conserved N-myristylation motif was identified for AHSV (amino acid 60-65 or 59-64). Distal to the myristylation motif is a region of positively charged residues. Both these features are located within the conserved domain (amino acid 43-92) previously reported by Van Staden *et al.* (1995). Upon comparison of other orbivirus NS3 proteins it was observed that the N-myristylation motif was highly conserved in the amino terminal region of all these proteins. The motif was located between amino acids 66-71 for BTV, amino acids 47-52 for CHV (Chuzan virus, a member of the

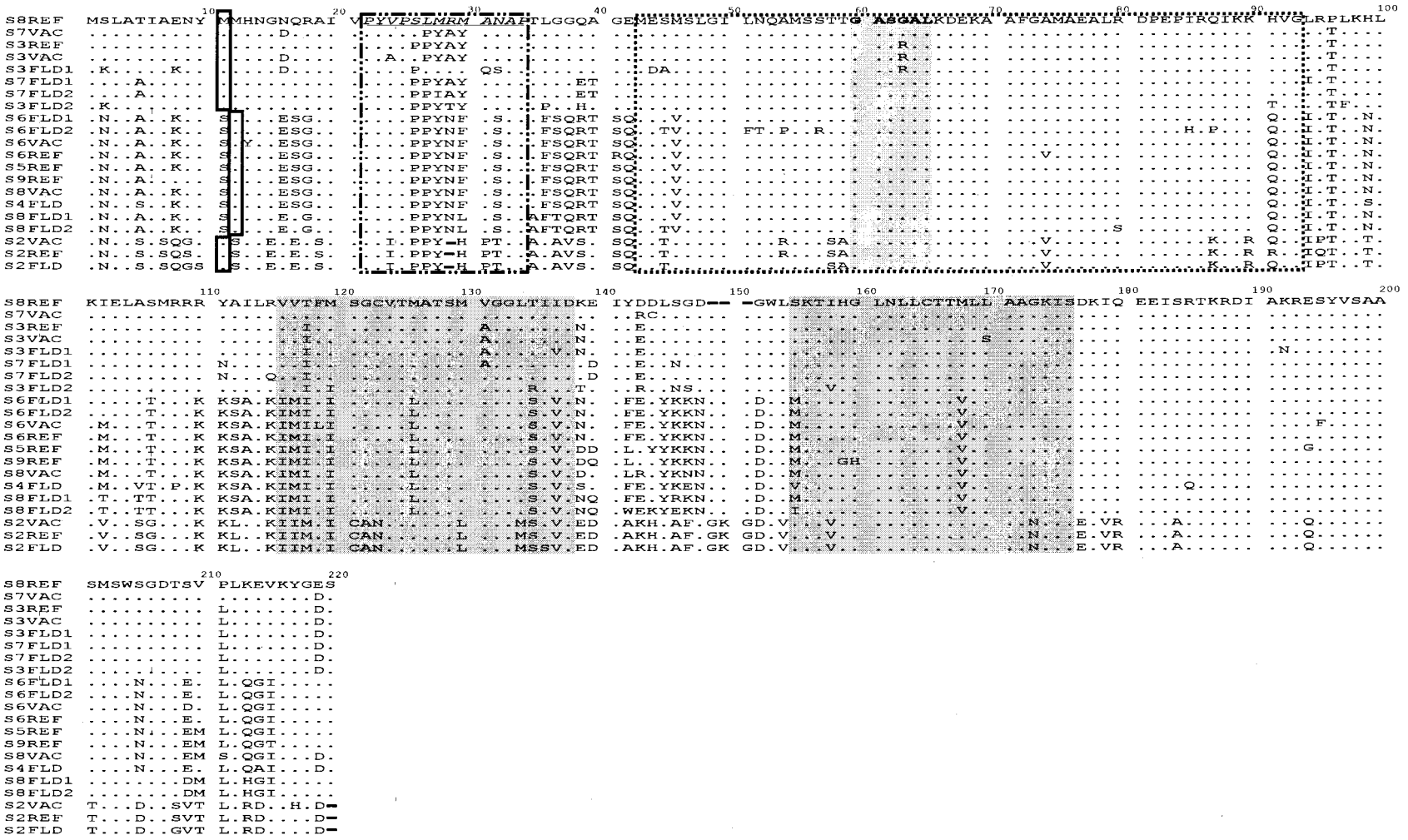


Fig 3.1 AHSV NS3 amino acid sequence alignment comparisons. Dots indicate identity to AHSV-8 reference strain NS3. The NS3A start codon is blocked, the proline rich region is underlined and blocked (amino acids 22-34). The highly conserved region is blocked (amino acids 43-93); the myristylation motif within this region is indicated by bold type and the corresponding sequences in the other AHSV NS3 proteins are shaded light grey. The two hydrophobic domains (amino acids 116-146 and 164-186) are shaded in dark grey.

Palyam serogroup), amino acids 62-67 for EHDV (Epizootic haemorrhagic disease virus) and amino acids 37-42 for BRDV (Broadhaven virus) (Fig 3.2). These myristylation motifs were in all cases followed by a number of basic amino acids that have a random order as seen for AHSV NS3.

3.3.2 Variation level of AHSV NS3

To investigate the variation between the NS3 sequences of S.A. AHSV isolates the S10 gene sequences of 19 AHSV isolates were determined, translated into amino acid sequences and aligned (Fig 3.1). The percentage amino acid sequence variation within the NS3 proteins of AHSV and a number of other orbiviruses was subsequently investigated with an inferred distance matrix (Fig 3.3).

These results were compared to the percentage of NS3 variation previously reported in BTV, the percentage variation previously reported within rotavirus NSP4 and the percentage variation within other AHSV proteins that was determined in this study using Genbank deposited sequences (Table 3.3). Distance scores from the inferred distance matrixes, summarized as histograms (Fig 3.4), indicated that AHSV NS3 varied by as much as 27.6% (between S8FLD2 and S8REF) within a serotype and up to 36.3% (between S8REF and S2VAC or S2FLD) amongst different serotypes (Fig 3.4A). This is in strong contrast to the 7% variation of BTV NS3 (Hwang *et al.*, 1992*a*). The cognate protein in rotaviruses, NSP4, varies by 19.4% (Kirkwood & Palombo, 1997) which is significantly less than the AHSV NS3 protein (Fig 3.4A).

The NS3 sequences of the virulent field strains were compared to the avirulent vaccine strain NS3 sequences of the corresponding serotype. The amount of variation was found to be 2.3% for AHSV-2, 7.4% - 8.8% for AHSV-3, 2.3% - 6.0% for AHSV-6, 6.0% - 6.5% for AHSV-7 and 7.8% - 9.7% for AHSV-8 (Fig 3.4A). With the exception of AHSV-8 vaccine and reference strains (26.2%

variation), comparisons of NS3 sequence variation of vaccine and reference strains within a particular serotype ranged between 1.8% - 3.2%. The AHSV-2 vaccine strain was produced from the reference strain 82/61. It is unknown if the AHSV-3 and -6 vaccine strains are attenuated forms of the respective reference strains used in this study. The high level of NS3 sequence variation between the AHSV-8 vaccine and reference strains suggests that the vaccine strain of AHSV-8 was not directly derived from the AHSV-8 reference strain analyzed in this study. The specific variation between virulent field isolates within AHSV-3, -6, -7 and –8 ranged from 2.3% (AHSV-7 field isolates) to a maximum of 11.1% (AHSV-3 field isolates). The AHSV-3 field isolates displayed the highest variation and originated from geographically distinct areas and were isolated in different years i.e. Gauteng (S3FLD1) in 1997 and Kwa-Zulu Natal (S3FLD2) in 1998. The field isolates of AHSV-6 differed from one another by 3.7% and were isolated from different geographic regions in South Africa (Kwa-Zulu Natal and Free State) in the same year. The field isolates with the least variation i.e. AHSV-7 (2.3%) and AHSV-8 (2.8%), were all isolated from the same geographic area (Gauteng) in the same year.

BTV NS3	*GATQT*^{70}**Q**KAEKAAFAS^{80}YAEAFRDDVR^{90}RQIKRHVNE^{100}QILPLKLSDL^{110}GGLKKKR
EHDV NS3	*GATMAQ*VE^{70}KVAYASYAEF^{80}RDDLRLRQIK^{90}KHVNEQILPK^{100}MRVELTAMKR^{110}RR
AHSV NS3	*G*^{60}*ASGAL*KDEKA^{70}AFGAMAEALR^{80}DPEPIRQIKK^{90}HVGLRPLKHL^{100}KIELASMRRR^{110}
CHV NS3	*GVLN*^{50}*NA*MNDTTAAT^{60}QAEREEKVAY^{70}ASFAEALRDS^{80}ACVREIKKRV^{90}SSRTIIALEK^{100}EYNHQRR
BRDV NS3	*GTGA*^{40}*NE*VMRNEKAA^{50}YGAATVLKDD^{60}ETTRMLKMQV^{70}NEYSLAEMRA^{80}AYKLKK
HIV Gag	M*GARASV*LSG^{10}GELDRWEKIR^{20}LRPGGKKKKYK^{30}LKHIVWASRE^{40}

Fig 3.2 Proposed orbivirus NS3 myristylation motif. The bipartite signal consists of a myristylation motif (underlined in italics) followed by a number of basic residues (bold type). The similar membrane targeting signal identified for HIV-1 Gag (Zhou *et al.*, 1994) is shown.

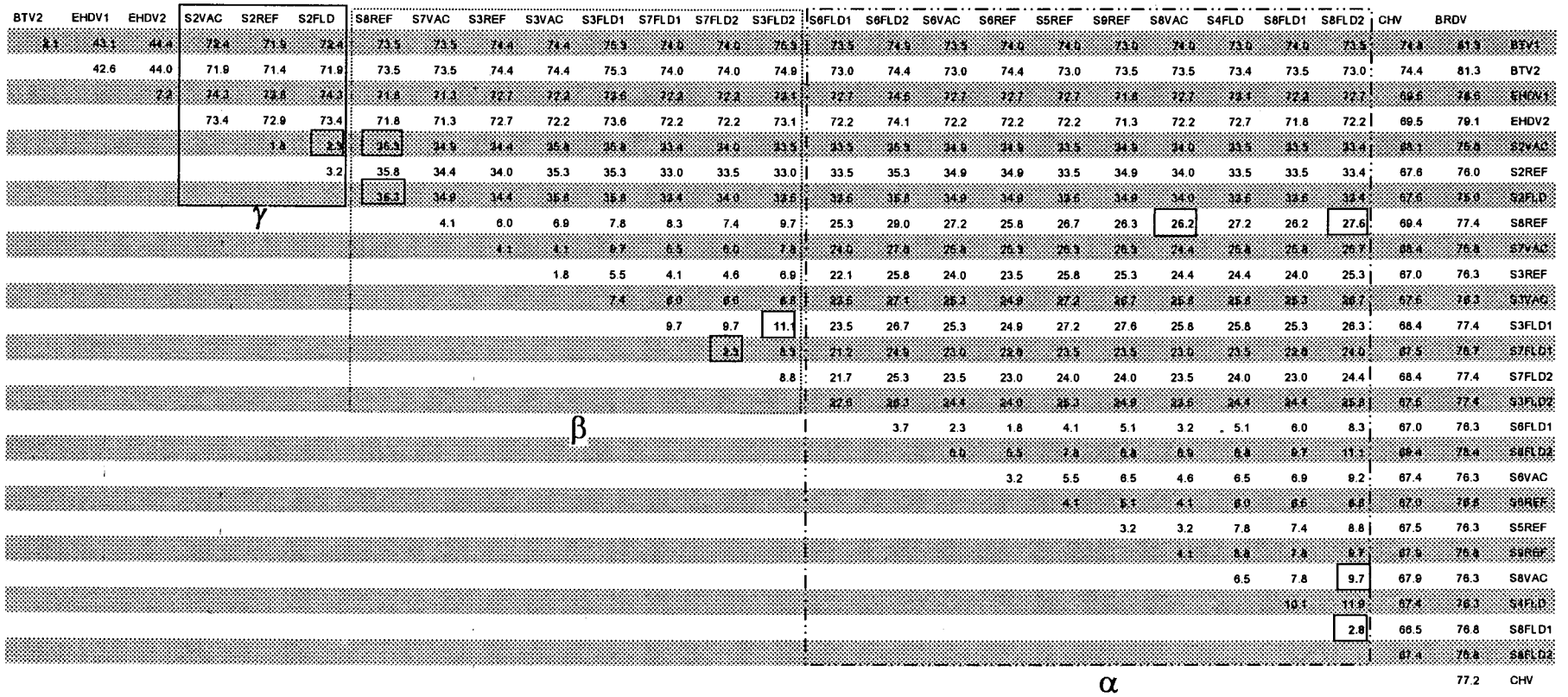


Fig 3.3 Distance matrix showing the percentage amino acid differences between AHSV NS3 sequences of this study, BTV NS3, EHDV NS3, CHV NS3 and BRDV NS3. AHSV NS3 phylogenetic clusters α, β and γ are indicated by large blocks and percentages of NS3 variation frequently referred to in the text are blocked.

Sequence alignment of orbivirus NS3 proteins indicated that AHSV NS3 differed by a maximum of 69.4% from CHV NS3, by 74.5% from EHDV NS3 and by 75.3% from BTV NS3. BRDV NS3 was found to be the most divergent NS3 protein in the orbivirus group.

AHSV NS3 variation was furthermore compared to that of other AHSV proteins. To compare the level of AHSV NS3 variation with that of the other AHSV proteins the variation of VP2, VP3, VP5, VP6. VP7 and NS1 was determined. In comparison to other AHSV proteins (Fig. 3.4B), NS3 is the second most variable virus protein with approximately 20% less variation than the observed 56% variation between the outer capsid proteins (VP2) of different serotypes. Other AHSV proteins (VP3, VP5, VP6, VP7 and NS1) have an internal variation ranging between 19% (VP5) and 0.2% (VP7). As expected the sequence conservation of the inner core proteins (VP3 and VP7) is significantly higher than that of the outer capsid proteins (VP5 and VP2).

3.3.3 The possible use of NS3 as an AHSV virulence marker

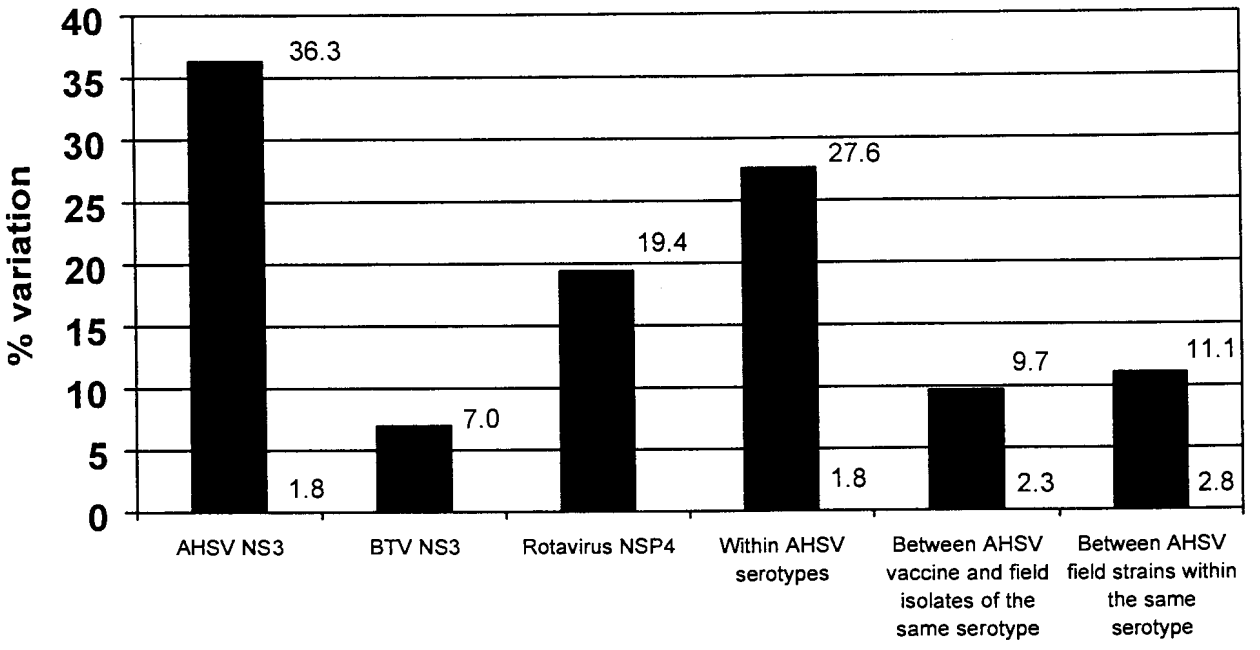
To identify possible AHSV virulence markers, the NS3 sequences of the virulent field and reference isolates were compared to the corresponding avirulent vaccine strain NS3 sequences of serotypes 2, 6 and 8. An amino acid substitution is only likely to have a significant influence on the protein's functional properties if the new amino acid has different biochemical properties to the amino acid that it has replaced. When the AHSV-2 vaccine and virulent strain NS3 sequence were compared, only one unique and significant amino acid substitution was identified in position 217 of the alignment (Fig 3.1). The AHSV-6 vaccine strain NS3 protein also had one unique and significant amino acid change in position 13 of the alignment while another single unique and significant amino acid difference was located at position 211 for the AHSV-8 vaccine strain NS3. The details of the amino acid changes and the probable consequences of these changes are given in Table 3.4. None of these amino acid changes were

located in the hydrophobic domains of NS3, implicated to be involved in the cytotoxicity of NS3. These changes were further not located within any of the other conserved domains of AHSV NS3. The only common factor between these mutations is that they were all situated in the terminal regions of the protein. The absence of a consensus sequence marker in NS3 makes it unlikely that these substitutions represent a definitive virulence marker for AHSV.

Table 3.4 Significant amino acid differences in the NS3 sequence of AHSV vaccine strains in comparison to virulent AHSV strains.

AHSV serotype	Amino acid position as indicated in Fig 3.1	Amino acid substitution	Possible significance in NS3
2	217	Tyr to His	Change in charge
6	13	His to Tyr	Change in charge
8	211	Pro/Leu to Ser	Change in chemical nature

A



B

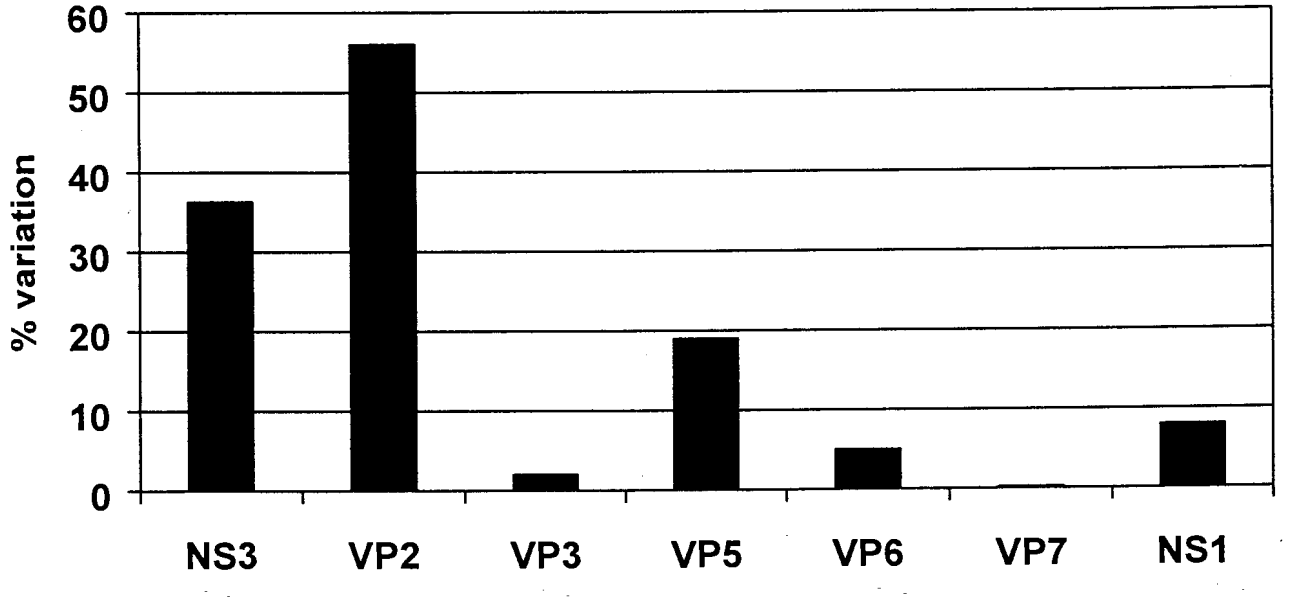


Fig 3.4 Comparative variation of AHSV NS3. (A) The total level of variation of AHSV NS3 across serotypes compared to that of BTV NS3 and rotavirus NSP4. The internal level of AHSV NS3 variation within serotypes, between vaccine and virulent field strains of the same serotype and between field strains of the same serotype are shown. Maximum and minimum variation percentages are indicated to the right of the bars. (B) The level of AHSV NS3 variation compared to that found in other AHSV encoded proteins.

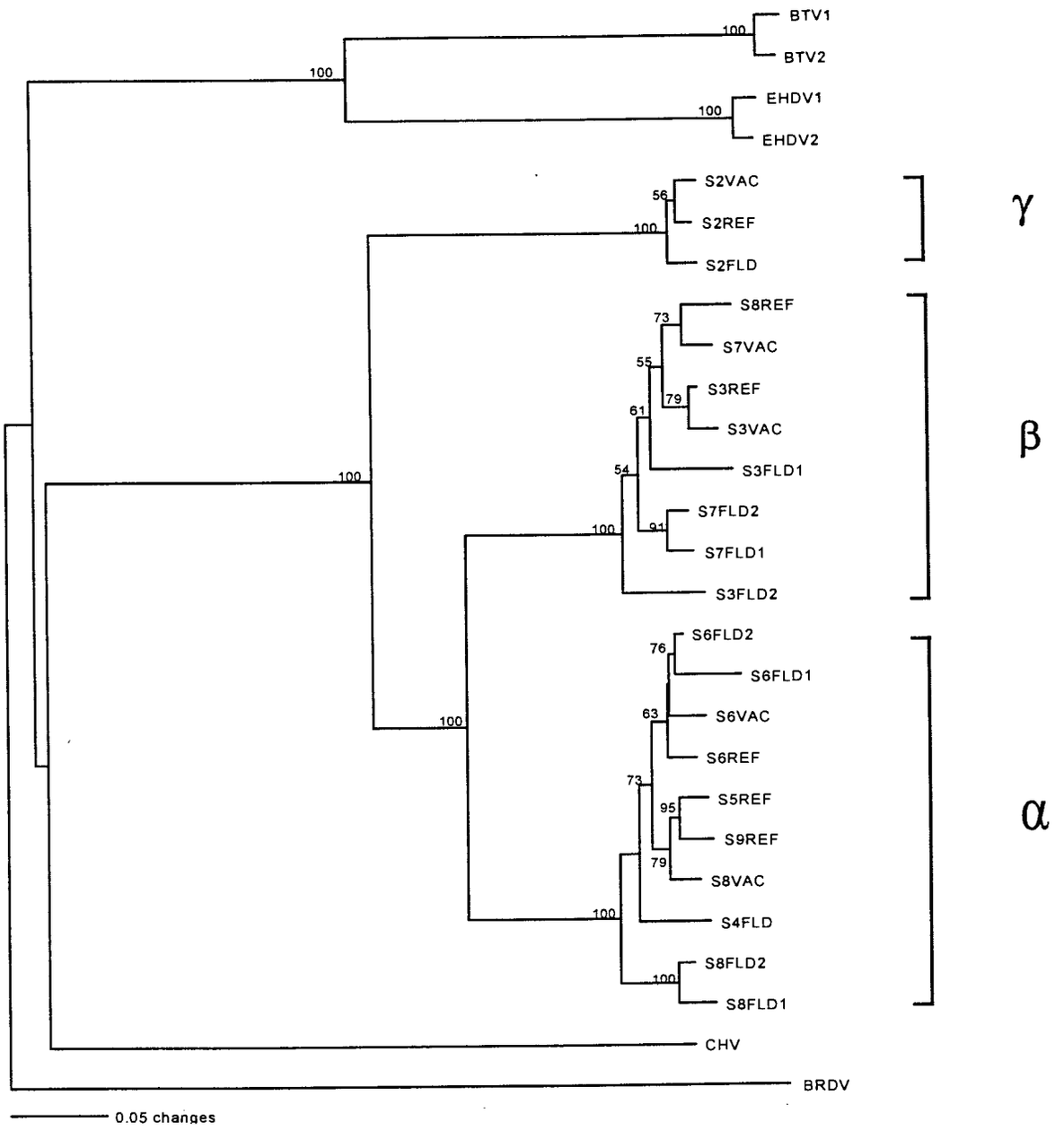


Fig 3.5 Phylogenetic tree of AHSV NS3 and other orbivirus NS3 protein sequences. The horizontal branch lengths are indicative of the genetic distance between the sequences. The tree was constructed using the Neighbour joining method (PAUP ver 4.0b3) and bootstrapped with 1000 replicates. Confidence levels are as indicated.

3.3.4 AHSV NS3 Phylogeny

To investigate the phylogeny between virulent field isolates, vaccine strains and laboratory reference strains, the aligned NS3 amino acid sequences were used to construct phylogenetic trees (PAUP ver 4.0b5) (Fig. 3.5). The estimated value of the gamma shape parameter was 0.58 and the small distances obtained using this value in a gamma test indicated that S10 was subject to unequal rates of mutation. Certain algorithms used to construct phylogenetic trees such as that used by the unweighted pairgroup method with arithmetic mean (UPGMA) assume equal rate of mutation and were therefore not used in this analysis. Results of the aligned S10 gene nucleotide sequences and inferred phylogenetic trees generated the same results as found for the NS3 protein sequences and are thus not shown. The NS3 proteins of related orbiviruses retrieved from Genbank (Table 3.3) namely BTV, EHDV, CHV and BRDV (selected as outgroup) were included for comparative purposes.

Various methods of tree construction all showed similar internal grouping of AHSV NS3. NS3 grouping of the related orbiviruses were as distinct clusters with a difference in the placement of CHV NS3 depending on the method of tree construction used. CHV NS3 grouped closer to AHSV when analyzing the data with neighbor joining while it grouped closely with BTV and EHDV when using parsimony. The bootstrap value of the node that connected CHV and AHSV NS3 in the neighbour joining tree was 53% indicating a low confidence for the grouping in this branch. However, a high bootstrap value (>90%) for the CHV and BTV branch with parsimony suggests that CHV NS3 is closer related to the BTV and EHDV NS3 sequences than to NS3 of AHSV.

The inferred phylogenetic trees of the S.A. AHSV isolates also indicated the presence of three distinct NS3 phylogenetic clusters as previously described viz., α, β and γ (Martin *et al.*, 1998; Sailleau *et al.*, 1997) with AHSV-4, -5, -6, -8 and -9 clustering in α, AHSV-3, -7 and -8 in β and AHSV-2 in γ. The α and β clusters

were found to share the most recent common ancestor and are therefor closer related to one another than to the γ cluster of AHSV-2 viruses. NS3 of the AHSV-8 isolates that we studied clustered into two different phylogenetic groups (α and β) and did not cluster with AHSV-2 (in γ) as reported previously. The AHSV-8 field and vaccine strains grouped with AHSV-4, -5, -6 and -9 (α), while the AHSV-8 reference strain grouped with AHSV-3 and AHSV-7 (β). The γ cluster showed that the AHSV-2 reference and vaccine strain NS3 proteins are closer related to one another than to NS3 of the AHSV-2 field strain. The grouping pattern of the β cluster indicated no sub-clustering of NS3 based on AHSV serotype. NS3 of AHSV-3 vaccine and reference strains were sisters while the NS3 of AHSV-7 vaccine and AHSV-8 reference strains were closely related. The AHSV-7 field isolates were closely related while NS3 of AHSV-3 field isolates did not share the same common internal node in the tree and are therefore more distantly related. The largest cluster in this study, α, grouped NS3 of all the AHSV-4, -5, -6, and -9 isolates and three of the AHSV-8 isolates together. The AHSV-6 NS3 proteins were found to form a distinct lineage within this cluster. The AHSV-6 field isolates were closely related to one another and the AHSV-6 vaccine and reference strains were sisters in this sub-cluster. The AHSV-8 NS3 proteins were more dispersed through this cluster. The AHSV-8 field isolates grouped in a separate lineage to that of the AHSV-8 vaccine strain that was in turn closely related to the AHSV-5 and -9 reference strains.

Since the AHSV-8 NS3 proteins clustered into alternate clusters (α and β) to that previously described (γ) it was necessary to confirm the serotype of the strains used. A useful and rapid means to differentiate between AHSV-8 and other AHSV serotypes is the smaller size of the AHSV-8 outer capsid protein VP2 in comparison to that of the other AHSV serotypes (O. Potgieter, personal communication). Viral dsRNA for the serotype 8 viruses was *in vitro* translated and analyzed on a large 15% polyacrylamide gel that had been electrophoresed overnight at 100 V. The protein gels were dried and exposed to auturadiographic film that was developed after 2 – 3 days using standard procedures. The

resultant autoradiograph clearly showed the entire protein profile of the AHSV encoded proteins (Fig 3.6). VP2 of the AHSV-8 strains (lane a), the second largest viral encoded protein, was smaller compared to that of the control AHSV-6 VP2 (lane b). This therefor confirmed the serotype of the AHSV-8 isolates.

The phyolgenetic analysis of the AHSV NS3 sequences was found to be useful as an epidemiological marker for AHSV outbreaks. This was demonstrated by the field isolates that grouped according to origin of geographic location. In was also clear that the vaccine strains were not closely related to any of the field isolates indicating that the vaccine strains were not the cause of the outbreaks that were studied here.

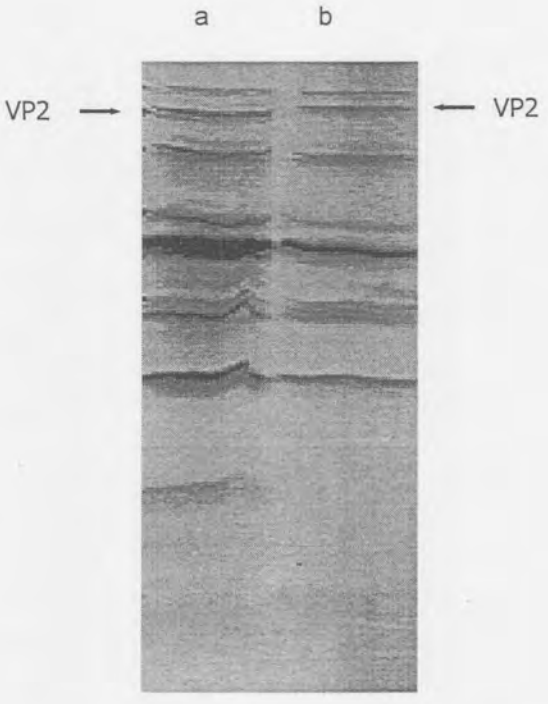


Fig 3.6 Autoradiograph of AHSV dsRNA *in vitro* translations indicating the size difference of AHSV-8 VP2 (lane a) compared to AHSV-6 VP2 (lane b).

3.3.5 BTV S10 and NS3 sequence analysis

Most of the BTV NS3 sequences have been carried out with isolates first isolated in diverse geographic locations including Australia, U.S.A. and China (Bonneau *et al.*, 1999; Pierce *et al.*, 1998: Hwang *et al.*, 1992*a*). Very little sequence data on the S10 gene and encoding NS3 protein of S.A. BTV strains is available.

This study included 9 recent S.A. BTV field strains isolated in 1999 of serotypes 1, 2, 3, 4, 8, 11 and 18, while an additional 12 strains were historical reference and vaccine strains dating back to as early as 1900 (Table 3.2). The S10 ORF of the S.A. BTV isolates all encoded a protein of 229 amino acids, unlike the cognate gene in AHSV that encodes for a protein of either 217 or 218 amino acids in length. The S.A. BTV NS3 sequence alignment is given in Fig 3.7. The second in phase methionine is conserved and located at position 14 of the full length NS3 protein. The proline rich region of BTV NS3 (position 36 – 49) was also a highly conserved feature in the S.A. BTV NS3 proteins. The region between residues 49 and 98 was highly conserved as previously reported (Van Staden & Huismans, 1991). This region contained a potential glycosylation motif (position 63 – 65) and a putative myristylation site followed by a highly positively charged region as described for AHSV NS3 and other orbivirus NS3 proteins. Two hydrophobic domains, proposed to be membrane-spanning regions (Van Staden, 1993; Bansal *et al.*, 1998), were highly conserved. Only one of the cysteine residues was highly conserved located at position 137. Another cysteine residue at position 181 was common in 18 of the 21 BTV NS3 proteins, the exceptions being S3VAC and S8F isolates that had a positively charged lysine in this position. Cys^{181} is located within a proposed membrane-spanning region, making it unlikely that the residue would be available for disulphide bond formations. The three BTV-11 isolates had an additional cysteine residue at position 39. The glycosylation motif at position 150–153 was conserved amongst all the isolates and this site has been shown to be glycosylated for BTV NS3

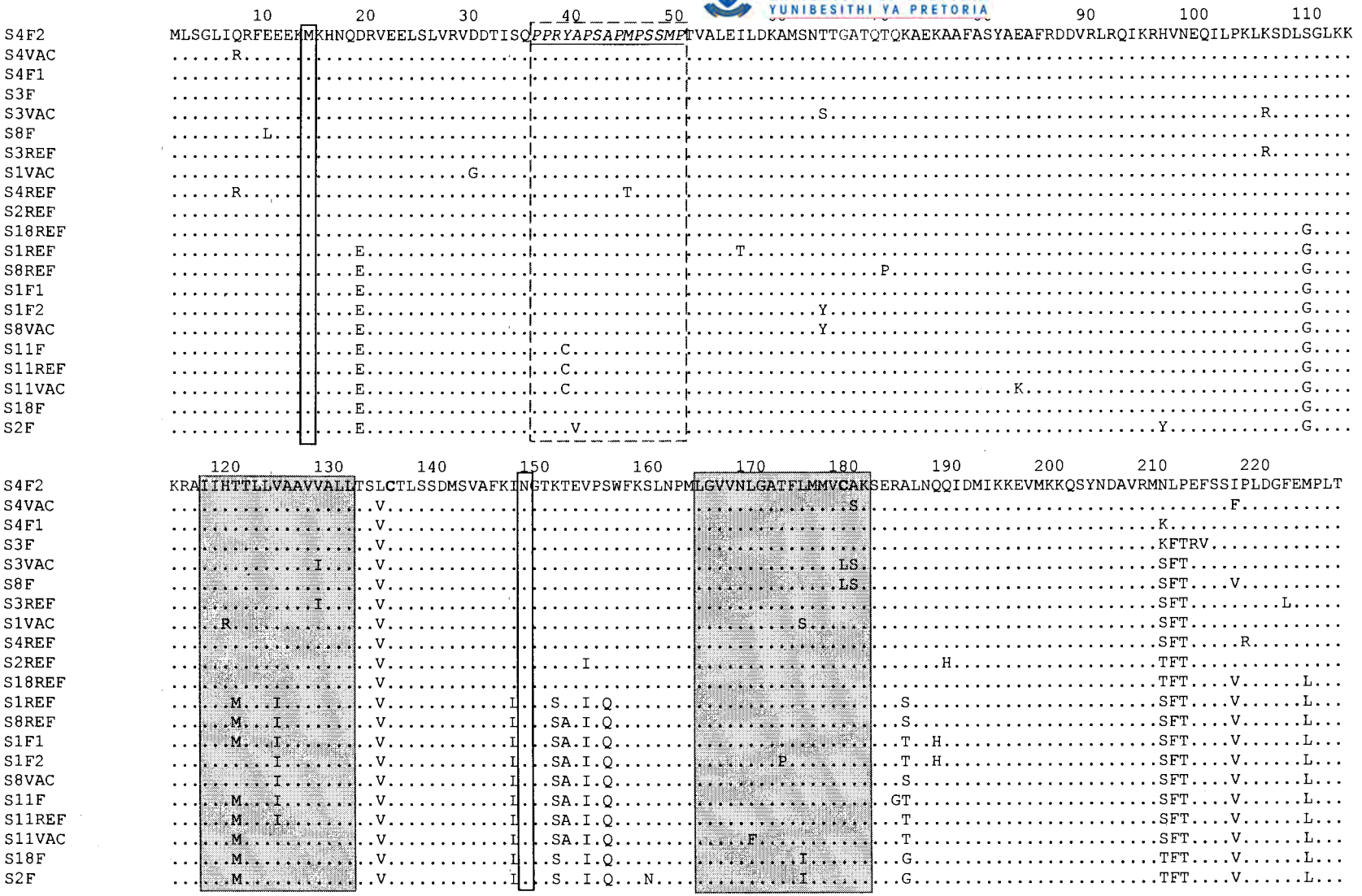


Fig 3.7 NS3 amino acid sequence alignment of S.A. BTV isolates. Dots indicate identity to NS3 of the BTV-4 Field strain, S4F2. The NS3A start codon is blocked, the proline rich region is underlined and blocked (amino acids 36-50). The two hydrophobic domains (amino acids 119-134 and 166-183) are shaded grey. The N-linked glycosylatation site (150) is blocked.

(Bansal *et al.*, 1998). The most variable regions within the protein were located between the two hydrophobic domains (approximately 25% variation between positions 142–161) and in the final 20 residues of NS3 (positions 211–226).

3.3.6 Variation level of BTV S10 and NS3

It was of interest to investigate the level of genetic variation in the S10 gene and NS3 protein of S.A. BTV isolates and to compare the variation level to that of international BTV S10 and NS3 sequences as well as to that of AHSV NS3 sequences. Previous BTV sequence variation studies (Bonneau *et al.*, 1999; Pierce *et al.*, 1998) have shown that BTV NS3 is highly conserved (about 7% amino acid variation). The percentage variation between the S.A. BTV S10 genes (Fig 3.8) and encoding NS3 proteins (Fig 3.9) was determined. The highest S10 nucleotide sequence variation of 20% (Fig 3.8) was found between the S8REF (1937) and S4VAC (1900) isolates. The maximum NS3 protein variation (Fig 3.9) that was found between numerous isolates reached 9%. The greatest level of NS3 sequence variation within a serotype (7%) was between the BTV-1 vaccine and reference strains both isolated in 1958. The intra-serotype variation levels for vaccine and reference strain NS3 sequences ranged between 1 and 3% for serotypes 3, 4, 8 and 11. The serotype 1 and serotype 8 BTV field isolates NS3 sequences differed from the respective vaccine strain NS3 sequences by 7%, the serotype 4 vaccine and field isolates differed by 1% and the serotype 3 vaccine and field isolates differed by 4%. The BTV and EHDV NS3 proteins in this analysis differed by a maximum 47%.

	S8VAC	S8REF	S2F	S11F	S11VAC	S11REF	S1REF	BTV-11	BTV-17V	S1F1	S1F2	BTV-16	S18F	BTV-12	BTV-15	S4VAC	S4REF	S3F	S4F2	S4F1	S18REF	S2REF	S8F	S3REF	S3VAC	S1VAC	BTV-13F	BTV-17F	EHDV-1	EHDV-2	AHSV-3	AHSV-8
S8VAC																															
S8REF	1																														
S2F	8	8																													
S11F	8	9	4																												
S11VAC	8	8	6	6																											
S11REF	7	7	6	5	1																										
S1REF	8	7	6	7	8	8																									
BTV-11	8	8	10	9	8	8	9																								
BTV-17V	8	8	10	9	8	8	9	2																							
S1F1	17	16	17	16	17	17	16	17	17																						
S1F2	17	16	17	16	17	17	16	17	17	0																					
BTV-16	6	15	16	16	16	16	15	16	16	3	3																				
S18F	17	16	16	16	16	16	15	16	16	4	4	3																			
BTV-12	17	17	17	17	17	16	17	16	16	9	9	9	9																		
BTV-15	17	16	17	16	15	15	17	15	14	13	13	13	13	13																	
S4VAC	19	20	18	18	19	19	18	19	18	18	18	18	17	17	18																
S4REF	19	20	19	19	19	19	19	19	18	18	19	18	17	18	18	2															
S3F	18	19	18	18	18	18	16	18	17	17	17	17	16	18	18	5	5														
S4F2	18	19	18	18	18	18	17	18	17	18	18	17	16	18	17	5	5	1													
S4F1	18	18	17	18	18	18	16	17	17	16	16	16	15	17	16	5	6	2	3												
S18REF	8	19	17	17	18	18	17	18	17	17	17	18	18	17	18	5	6	4	4	3											
S2REF	17	18	16	17	17	17	17	17	17	17	17	16	16	17	17	5	5	3	4	4	3										
S8F	18	19	18	18	18	18	18	17	18	18	18	17	17	18	18	7	7	4	6	5	5	5									
S3REF	18	19	18	18	18	18	17	18	17	18	18	17	17	18	18	6	6	5	5	6	6	4	6								
S3VAC	18	20	19	18	18	18	18	19	18	19	19	18	18	19	19	7	7	7	7	7	7	5	5	1							
S1VAC	18	18	18	18	18	18	16	18	17	17	17	17	18	17	17	9	9	8	8	8	8	8	8	7	7						
BTV-13F	18	19	18	19	18	18	18	18	17	17	18	17	17	18	17	8	8	8	8	8	8	7	8	6	7	8					
BTV-17F	17	18	18	18	18	18	17	18	17	18	18	17	17	17	17	8	7	8	7	8	8	7	8	5	7	8	2				
EHDV-1	38	38	39	40	38	40	40	38	37	40	40	39	39	40	40	39	40	38	39	38	38	38	39	38	38	37	37	38			
EHDV-2	39	38	40	40	40	40	40	38	38	40	40	40	39	40	40	40	40	39	40	39	39	38	40	38	38	38	38	39	4		
AHSV-3	55	55	56	55	55	55	55	55	55	55	55	55	55	53	54	56	57	55	55	56	55	55	56	57	57	56	56	55	56	56	
AHSV-8	54	54	53	53	54	54	54	53	53	54	54	55	55	53	54	56	56	55	55	56	55	55	56	56	56	56	56	56	57	56	26
AHSV-2	54	54	54	53	54	53	54	51	54	55	55	55	55	54	55	55	55	56	55	55	54	55	56	54	54	54	54	54	58	58	34	31

Fig 3.8 Distance matrix showing the percentage nucleotide differences between the S.A. BTV, AHSV and EHDV S10 genes inferred using the uncorrected 'p' distance setting (PUAP ver 4.0b3). Variation percentages referred to in the text are in bold type-script and blocked.

	S4VAC	S4F1	S3F	S4REF	BTV-17F	BTV-13	S1VAC	S3VAC	S8F	S3REF	S2REF	S18REF	S8REF	BTV-17V	BTV-11	S8VAC	S1F2	S1F1	S11F	S11REF	S11VAC	S1REF	S18F	S2F	EHDV-2
S4F2	2	1	3	3	2	2	3	4	4	3	3	5	7	7	7	7	8	7	8	7	8	7	7	8	46
S4VAC		2	4	3	3	3	4	5	4	4	4	4	8	8	8	8	9	8	9	8	9	7	7	9	47
S4F1			2	3	1	2	3	4	3	3	2	3	7	7	7	7	7	7	7	7	7	7	6	7	46
S3F				3	1	2	3	4	3	3	2	3	7	7	7	7	7	7	7	7	7	7	6	7	46
S4REF					1	2	3	4	3	3	3	3	7	7	7	7	7	7	7	7	7	7	7	8	46
BTV-17F						0	1	3	2	1	1	2	6	5	5	5	6	6	6	6	6	5	5	7	45
BTV-13							2	3	3	2	2	2	6	6	6	6	7	6	7	6	7	6	6	7	45
S1VAC								4	3	3	3	3	7	7	7	7	7	7	7	7	7	7	6	7	45
S3VAC									2	2	4	4	8	8	8	7	8	8	9	8	9	8	8	9	46
S8F1										3	3	3	7	7	7	7	7	7	7	7	7	7	7	8	47
S3REF											3	3	7	7	7	7	7	7	7	7	7	7	7	8	45
S2REF												2	6	6	6	6	7	6	7	6	7	6	7	6	45
S18REF													6	6	6	5	6	6	6	6	6	6	4	6	46
S8REF														0	1	1	3	1	2	1	3	1	3	4	44
BTV-17V															0	1	2	1	1	1	2	1	2	3	44
BTV-11																1	3	1	2	1	3	1	3	4	44
S8VAC																	1	2	2	2	3	2	3	4	44
S1F2																		1	3	2	3	3	4	5	44
S1F1																			1	1	2	2	3	4	44
S11FREF																				0	2	2	3	4	44
S11FREF																					1	2	3	4	44
S11VAC																						3	3	4	46
S1REF																							2	3	44
S18F																								1	45
S2F																									46
EHDV-2																									

Fig 3.9 Distance matrix of S.A. BTV NS3 proteins showing percentage amino acid differences between the S.A. BTV NS3 sequences, some U.S.A. BTV NS3 sequences and an EHDV NS3 sequence. Variation percentages referred to in the text are in bold type-script and blocked.

3.3.7 A comparison of the NS3 sequence of BTV strains first isolated at very different times in the last century

This study included historical BTV strains dating back to the early 1900s and recent field isolates from 1999. This created a possible opportunity to study any random mutation events in S10 over time. The full length BTV S10 ORF alignment (691 characters in nucleotide alignment data set, not shown) had 164 variable sites or variation over 23% of the nucleotide sites for the isolates in this study. A total of 16% of these sites showed mutations in the degenerate position that would lead to a silent mutation with no change in the encoding amino acid. It was found that 84% of these 164 sites had mutations common to both the historical and recent BTV isolates indicating that these mutations were not recent but part of the existing pool of BTV S10 variation. Certain mutations were, however, exclusively found in either the historical or recent BTV isolates. Interestingly, both historical and recent isolates had unique S10 mutations in approximately 18% of the total mutated sites. These percentages are not additive. This can be explained as a quarter of the variable sites had more than two different nucleotide substitutions present that were subsequently analyzed as separate mutation events for the respective virus clusters. This may indicate that the recent field isolates had introduced some new mutations into the pool of S10 variation. Conversely, several of these mutations may be found in other historical isolates not analyzed in this study with the pool of variation thereby remaining relatively constant. The specific NS3 sequence variation between the serotype 3 (1944) historical and 1999 field isolates was 4%, between the serotype 4 historical (1900) and 1999 field isolates 2–3% and the serotype 11 historical (1944) and 1999 field isolates 2%. The S10 sequence variation ranged between 4% and 7% for the isolates in the above-mentioned BTV serotypes. This analysis did not indicate a great divergence in the S10 gene sequences of these S.A. BTV isolates over the 100 year period and confirms that BTV S10 is strictly conserved.

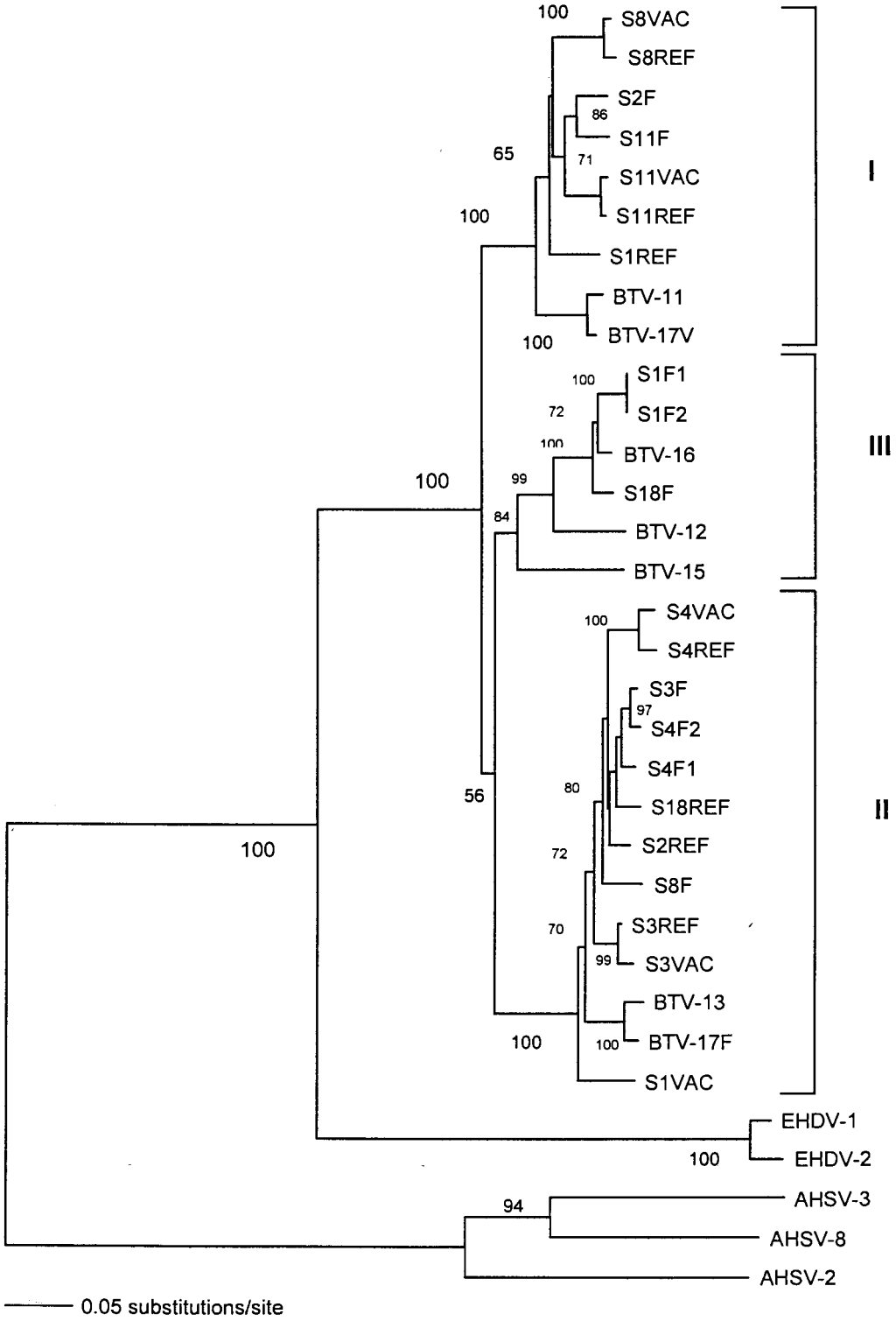


Fig 3.10 Phylogeny of S.A. BTV S10 genes. The phylogenetic tree was inferred using the Neighbour joining method with the HKY85 distance setting (PUAP ver 4.0b3) and bootstrapped with 1000 replicates. The branch lengths are indicative of the genetic distances between the sequences. The S10 genes of AHSV-2, -3 and -8 were included as outgroups in the analysis and the U.S.A. BTV, Chinese BTV and EHDV S10 genes were included for reference purposes.

3.3.8 BTV S10 phylogeny

The first aim of the BTV S10 analysis was to compare the level of variation found between the S.A. BTV isolates in relation to that of international BTV isolates. Secondly, it was of interest to investigate the phylogenetic relatedness of the S.A. BTV isolates, in particular that between the historical vaccine and reference isolates and the recent field isolates. The 21 BTV S10 genes and encoding NS3 proteins were compared and aligned with the S10 genes and NS3 proteins of AHSV and EHDV. The limited sequence variation in the NS3 protein sequences of the BTV isolates gave little phylogenetic resolution for studying the genetic relatedness of the S.A. BTV isolates. The higher level of BTV S10 gene variation was more robust to investigate the evolutionary relationship of these viruses.

Two methods of tree construction, namely neighbour joining (distance method) and parsimony, generated identical branching patterns when used to analyze the phylogeny of the isolates in this study. Bootstrap analysis (see values at branch nodes of trees) generally gave significant confidence levels (> 70%) for the trees validating the phylogenetic relatedness of the isolates. Both trees indicated that the BTV S10 genes were closer related to the S10 genes of EHDV than that of AHSV and that these different orbivirus genera formed distinct divergent groups. The BTV S10 genes grouped into three distinct clusters designated I, II and III for the purpose of further reference. Cluster II and III evolved from a more recent common ancestor and were closer related while cluster I diverged prior to the split between II and III. The bootstrap values for the branching of cluster I from clusters II and III was 100% while a much lower value (56%) was indicated for the divergence of clusters II and III (Fig 3.10).

Cluster I included S.A. virus isolates of serotypes 1, 2, 8 and 11 and serotypes 11 and 17 for the U.S.A. isolates (Pierce *et al.*, 1998). A total of 7 S.A. BTV isolates clustered into this group with 71% of the 7 being older vaccine and reference strains while 29% were recent field isolates. The highest amount of

variation in S10 for the S.A. isolates in this group was 8% found between the S1REF (1958) strain and the S8VAC (1937), S11VAC (1944) and S11REF (1944) strains. The S1REF strain varied by 9% from that of the U.S.A. isolates in this cluster. The serotype 11 vaccine and reference strains were the closest relatives to the field isolates in this cluster while the serotype 1 and serotype 8 isolates were members of two separate branches respectively. The vaccine and reference strain S10 genes for the serotype 8 and serotype 11 isolates were sisters on a distinct node indicating their close genetic relatedness and possibly that the vaccine strains were developed from the corresponding reference strain. However, the serotype 1 reference strain (1958) was not closely related to the vaccine strain produced in the same year (1958) which grouped into cluster II. Two U.S.A. BTV isolates, BTV-11 (1962) and BTV-17V, grouped with the S.A. cluster I viruses as taxa of a distinct subcluster that were not directly related to any of the S.A. BTV isolates.

Cluster II included 11 S.A. BTV isolates with 64% of these isolates being historical vaccine and reference strains while 36% were recent field strains. The highest level of S10 sequence variation (9%) in this cluster was between S1VAC and S4VAC as indicated in the distance matrix (Fig 3.8). The recent 1999 field isolates of serotypes 3, 4 and 8 clustered in group II. The BTV-3 and BTV-4 isolates grouped in a subcluster while the BTV-8 isolate formed a single taxa of another branch. The reference and vaccine strains in cluster II were not closely related to any of the recent field isolates. It was evident from the branching patterns that S4VAC was probably derived from S4REF and similarly S3VAC was derived from S3REF. The S1VAC, S2REF and S18REF strains were independent lineages in this cluster with no close relatives. Two older U.S.A. strains, BTV-13 (1989) and BTV-17F (1981) also grouped here. The U.S.A. BTV isolates formed a separate branch in this cluster but branched amongst the S.A. BTV isolates that indicated that the U.S.A. strains are related to the S.A. strains. In the study undertaken by Pierce and coworkers (1998) these two isolates were distantly related members of the same cluster.

The smallest cluster in this analysis, III, grouped three S.A. BTV field isolates (S1F1, S1F2 and S18F) together with the BTV strains that originated from China, namely BTV-12 (1996), BTV-15 (1996) and BTV-16 (1998) (Bonneau *et al.*, 1999). The BTV-1 field isolates were closely related while S18F and the Chinese strains were more distantly related and formed individual branches in this cluster.

From the phylogenetic analysis it is further evident that segment shifting is likely to occur in nature as any serotype can contain a S10 gene from any of the clusters. This also occurs in the case of AHSV and has been found in other BTV S10 epidemiological studies (Pierce *et al.*, 1998; Bonneau *et al.*, 1999). The historical strains did not from distinct, independent lineages but clustered amongst the recent field strains. This may indicate that very little mutations have occurred in the BTV S10 gene over the past 100 years in southern Africa and that there appears to be a pre-determined level of BTV variation and that the older S10 genes constantly re-emerge.

3.4 Discussion

The overall NS3 protein sequences of the AHSV isolates in our study varied by 36.3%, which is in strong contrast to the much lower NS3 sequence variation previously reported for BTV (7%) (Hwang *et al.*, 1992*a*) and also larger than the level of S.A. BTV NS3 sequence variation found in this study (9%). The variation is also greater than the variation in the NSP4 protein of rotavirus (19.4%) (Kirkwood & Palombo, 1997). Despite the extensive variation, higher order structures of AHSV NS3 predicted to be of importance, for example membrane anchoring domains, are highly conserved. A highly conserved myristylation motif was also identified within the conserved N-terminal region of the orbivirus NS3 proteins. Although this sequence is common to a vast number of proteins and, therefore, does not imply that a protein is myristylated, it is nevertheless of interest in view of the fact that a major function of protein acylation is membrane targeting and association. Myristylation alone does however not provide sufficient

energy to attach the protein to the phospholipid bilayer (McLauglin & Aderem, 1995). Membrane associated proteins of other viruses such as Gag of human immunodefiecency virus (HIV-1) (Zhou *et al.*, 1994) and Src of Rous sarcoma virus (Silverman & Resh, 1992) contain a region of basic amino acid residues that stabilize membrane interactions. We identified a similar bipartite motif in all orbivirus NS3 proteins investigated that may function as a membrane targeting signal of cleaved orbivirus NS3 proteins.

The observed large inter-serotype genetic variability of 27.6% for AHSV-8 is furthermore significantly larger than the highest inter-serotype variation of 15% previously documented for AHSV-4 (Sailleau *et al.*, 1997). The large inter-serotype variation of AHSV NS3 was found to be useful for distinguishing between sub-populations of the same serotype and therefor has value as an epidemiological marker. Of particular interest is to distinguish between vaccine and field isolates of the same serotype. With respect to those serotypes investigated as part of this study, the variation in the range of 2.3–9.7% is large enough to make such a distinction particularly when combined with phylogenetic analysis (Fig 3.5). The variation in NS3 sequence between different field isolates, such as the AHSV-3 field isolates in this study, was large enough to distinguish between sub-populations within a serotype. This could be of some advantage when outbreaks of the same serotype occur in different localities in the country and there is a request to link the outbreak to the transport of animals between these different regions.

The origin of the observed NS3 variation in AHSV is not clear and can include many different variables. For example the intermediate vector (*Culicoides imicola*) may tolerate a large amount of random variation in NS3 without an adverse affect on virion viability. BTV gene segments evolved independently of one another by genetic drift in a host specific fashion, generating quasispecies populations in both ruminant and insect hosts (Bonneau *et al.*, 2001). Bonneau *et al.* (2001) additionally report on a unique viral variant randomly ingested by *C.*

sonorensis that fixed a novel genotype by founder effect. The founder effect occurs during the sequential passage of BTV between its insect and ruminant hosts. NS3 is membrane associated and may be under some immunological pressure unlike the inner capsid proteins and other nonstructural proteins (NS1 and NS2) that remain within cells. The large variation in the NS3 region between the two hydrophobic membrane-spanning domains illustrates that this area is able to tolerate a large amount of variation. A particular intriguing question that remains to be investigated is why BTV NS3 seems to be so much more conserved. It has been proposed that variation of BTV NS3 is limited by structural constraints important for its function (Pierce *et al.*, 1998). Why these limitations do not apply to the same degree for AHSV NS3 remains uncertain.

This AHSV NS3 phylogenetic study confirms the grouping of the majority of AHSV NS3 proteins into three distinct phylogenetic lineages (Martin *et al.*, 1998). In exception, NS3 of four different AHSV-8 isolates in this study grouped in two separate clusters (β and α) and not together with NS3 of serotypes 1 and 2 in the γ cluster as predicted from the literature. Therefore, it may be assumed that although 3 distinct NS3 phylogenetic clusters are evident, the placement of a specific AHSV NS3 is not exclusively defined by the serotype. Reassortment of AHSV genome segments may be able to explain some of this large inter-serotype variation. Reassortment is a natural occurrence in the case of viruses with segmented genomes such as orthomyxo- and reoviruses. Multiple AHSV serotypes simultaneously present in zebras (Barnard, 1993) and mixed AHSV infections in horses could facilitate S10 reassortment between virus populations of different serotypes. The serotype groupings in the NS3 phenogroups (Fig 3.5) appears to indicate a certain tendency for serotypes (i.e. those within the same NS3 cluster) to exchange the NS3 gene more readily. These serotypes may further have a higher incidence of co-circulation. AHSV field isolates of the same season (usually commencing from early January to late May) and same serotype are closely related, while those of different seasons are more distantly related. The close relatedness of the NS3 sequence of viruses of the same serotype that

were isolated from nearby geographic locations agrees with generally accepted epidemiological principles. In no instance was NS3 of a vaccine strain identical to NS3 of field isolates of the same serotype.

The two hydrophobic regions of AHSV NS3 have been associated with the cytotoxic effect of this protein (Van Staden *et al.*, 1998). Any variation in these hydrophobic regions would therefore have the potential to abolish or alter the cytotoxic effect of NS3. This investigation did not show any differences between the hydrophobic domains of vaccine and field isolates that could be used as a virulence marker. The only unique and significant amino acid differences in the vaccine strains NS3 sequences as compared to the corresponding virulent NS3 sequences in this study were located in the terminal domains of NS3. These differences did not occur in a single site and were further not common to a specific domain. The actual significance of these mutations therefor requires further investigation that has been addressed in the following chapter. Avirulence in rotavirus infections can be associated with mutations in NSP4, in particular between amino acids 131 and 140 that mediates binding to another viral protein VP4 (Zhang *et al.*, 1998). Certain NSP4 amino acid sequences of virulent and attenuated virus pairs in group A and C rotaviruses differ by two to three amino acids (Chang *et al.*, 1999). These differences did not, however, provide unique markers for attenuation and further did not cluster in the VP4 binding domain. Another attenuated form of human rotavirus differed from the parental strain by a single (position 45) conservative amino acid substitution (Ward *et al.*, 1997). Attenuation of this particular strain appeared therefor to be unrelated to mutations of the NSP4 gene. Amino acid substitutions in NSP4 between rotaviruses derived from diarrheal and asymptomatically infected kittens failed to lend support to the contention that mutations in NSP4 played a significant role in virulence (Oka *et al.*, 2001). However, a single codon difference between the authentic sequence of rotavirus SA11 NSP4 and the commonly used NSP4 cDNA clone (amino acid 47) enhanced the authentic NSP4s cytotoxicity and calcium influx properties when expressed as a

baculovirus recombinant protein (Tian *et al.*, 2000). The significance that a single amino acid substitution may have on the virulence of a particular rotavirus strain can therefor not be underestimated.

This investigation is the first to study the variation in NS3 between recent virulent field isolates of AHSV and their relatedness to the current vaccine strains. Sequencing of the comparatively small S10 gene in outbreaks of the disease may provide significant epidemiological information. It may be possible to track the origin of an outbreak and our data also lends support to the ability to subtype virus populations within a given serotype. These applications are supported by preliminary, unpublished results on a recent outbreak of AHSV-7 in a region of South Africa (Western Cape) that has been free from AHSV for a large number of years.

The variation of BTV NS3 in this investigation indicated a slightly higher value (9%) than that previously described (7%) (Hwang *et al.*, 1992*a*). Despite the limited variation of BTV S10 it may still be possible to differentiate between vaccine and field strain of BTV that could therefor also have value as an epidemiological marker in the South African context.

This study grouped the S.A. BTV S10 genes into three distinct lineages. These three BTV S10 phylogenetic clusters have previously been described (Bonneau *et al.*, 1999). Two of these clusters group U.S.A. isolates together while the third cluster groups Asian isolates from China and Australia. The S.A. BTV S10 phylogenetic analysis indicated that all three clusters are currently circulating in S.A. This may imply that the BTV strains in the U.S.A. and China originated from the southern African continent at some stage in the past. The topology of U.S.A. BTV strains in cluster II suggests that they are closer related to the S.A. BTV strains than that of the cluster I U.S.A. BTV strains. This seems to imply that the cluster II strains circulating in the U.S.A. may have been introduced after that of the cluster I strains.

Cluster III in this analysis consists of three recent 1999 S.A. BTV field isolates and Asian isolates dating to 1996 and 1998. In this cluster the S.A. field isolates are scattered throughout and the Asian strains do not form a distinct lineage. This may suggest that BTV is a more recent introduction into China than it is in the U.S.A., were distinct lineages are already apparent. The inclusion of additional BTV strains may substantiate this further.

Phylogenetic analysis of other U.S.A. BTV isolates' proteins such as VP4 and VP6 indicated a similar evolutionary pathway for these two proteins which differed from the phylogeny inferred from VP7 sequences and indicates a different evolutionary pathway for VP7 (Kowalik & Li, 1991; Hwang *et al.*, 1992*b*; Huang *et al.*, 1993). The phylogeny of BTV NS3 in previous studies (Hwang *et al.*, 1992*a;* Pierce *et al.*, 1998; Bonneau *et al.*, 1999) and this study differs from that inferred for VP4, VP6 and VP7 indicating that BTV NS3 evolves independently of these proteins.

BTV NS3 also shows extensive shifting as the S10 clusters do not conform with BTV serotypes (Pierce *et al.*, 1998). The U.S.A. BTV isolates used in the study of Pierce *et al.* (1998) included serotypes 2, 10, 11, 13 and 17. Only serotype 2 was confined to a single cluster in their analysis. Analysis of S10 of the S.A. BTV isolates showed shifting of S10 between serotypes 1 and 2. The S.A. BTV isolates of serotypes 4, 3 and 18 clustered into group II while serotypes 8 and 11 clustered into group I. The restriction of these serotypes in a single cluster is likely to change with the inclusion of more S10 sequences of S.A. BTVs. In most instances the vaccine strains were directly related to the corresponding reference strains, the exception being the vaccine and reference strains of serotype 1, that in this study, were unrelated.

An interesting aspect of the BTV NS3 variation analysis was the inclusion of historical isolates, some of which dated back to the early 1900s. This enabled us to monitor the level of sequence variation between these historical strains and

some recent field isolates within and across the serotypes. The results suggested that little variation had accumulated over the last 100 years and that the S10 gene of BTV is therefor strictly conserved.

Limited NS3 sequence data of other orbiviruses restricts a comprehensive comparison of the typical variation for this nonstructural protein. Future work could focus on broadening the NS3 study to include more genera of orbiviruses.

The results in this chapter raised the question as to what the impact of these large AHSV NS3 sequence divergence levels could have on virus phenotypic characteristics. We therefor further investigated the impact of AHSV NS3 variation levels by selecting virus strains that differ significantly in NS3 sequence composition (i.e. that between the α, β and γ groups) as well as a vaccine strain. Parameters that encompass proposed NS3 associated functions such as cytotoxicity and virus dissemination were compared amongst these different strains.

CHAPTER 4

IMPACT OF AHSV NS3 VARIATION ON VIRAL PHENOTYPIC PROPERTIES AND CONTRIBUTION TO VIRULENCE

4.1 INTRODUCTION

The previous chapters focused on two characteristics of AHSV NS3, namely its membrane association and sequence variation. Membrane association assay results indicated that both hydrophobic domains (HDs) of AHSV NS3 are important for its membrane insertion and the resultant cytotoxic effect on infected cells. The subsequent sequence variation analysis investigated whether differences in virulence characteristics between attenuated vaccine strains and virulent field strains could be attributed to significant sequence changes in either of the HDs. No distinct sequence based virulence markers were identified. It was however evident that based on the high level of AHSV NS3 sequence variation, three distinct NS3 phenogroups could be distinguished. This raised the question if the large differences in NS3 sequence could be associated with phenotypic differences such as cytotoxicity and the release of virus particles from infected cells.

The precise function of NS3 remains unclear, but in the case of BTV there is good evidence that it is associated with virus release from infected cells (Hyatt *et al.*, 1993). In the case of AHSV it is associated with cellular membranes (Stoltz *et al.*, 1996) and the ability of AHSV NS3 to associate with membranes appears to be involved in its cellular cytotoxicity (Van Staden *et al.*, 1998 & see Chapter 2). However, the effective mechanism by which this association causes cell death is unknown. It may be related to a similar effect that viroporins have on cell membranes i.e. altering their permeability (Carrasco, 1994). AHSV NS3 has many properties in common with viroporins and this link would be strengthened if it can be shown that it alters the permeability of infected cell membranes. To investigate the possible functional significance that NS3 sequence variation may have on AHSV virulence properties the membrane permeabilization of AHSV vaccine and virulent strain's NS3 proteins were compared as well as functional differences between AHSV strains that encode different NS3 proteins.

In the case of AHSV-2 vaccine and virulent strains there are only four very specific amino acid differences between the NS3 proteins. It was of interest to

determine whether these amino acid changes, not located within any of the conserved domains of NS3, reflected on the virulence status of the virus i.e. vaccine strain being avirulent and the original field strain being virulent. To address this question, the AHSV-2 S10 genes were cloned, the NS3 proteins expressed and the effect of NS3 on the membrane permeability monitored.

The second objective was to investigate if a large difference in NS3 sequence variation (i.e. that between the α, β and γ phenogroups for example) could be associated with differences in virus phenotypic properties. To address this question AHSV isolates were selected and compared in terms of virus release, CPE and membrane permeabilization.

4.2 MATERIALS AND METHODS

4.2.1 AHSV titrations and plaque purification

Virus isolates were kept to a minimum passage number on Vero cells. For large scale production of virus stocks, Vero cells were infected with virus made at a MOI of 1 and harvested approximately 3 - 4 days p.i. when the cells reached a CPE of 3 (90% cell death). The virus stocks were collected, pooled and stored at 4°C.

Vero cells were maintained in Minimal essential medium (MEM) (Highveld Biological) supplemented with 5% FCS and antibiotics (60 mg/ml penicillin, 60 mg/ml streptomycin, 150 µg/ml Fungizone, all Highveld Biological). Cell monolayers were regularly divided by adding a small volume of Trypsin/Versene (Highveld Biological) to a monolayer pre-rinsed with sterile 1 x PBS. Once cells had separated from the flask surface, approximately 7 ml of supplemented MEM was added in which the cell clumps were dispersed by passing the suspension several times through a 15 G needle attached to a sterile syringe. The appropriate volume of culture medium was then added and the cells divided into the desired number of culture flasks.

Virus stocks, propagated from low MOI infections on Vero cells, were titrated to determine the virus titres. Six well plates were seeded with Vero cells and infected at approximately 80-90% confluency with 1 ml aliquots of virus stocks (diluted typically from 10^{-1} to 10^{-8} in MEM) and left to adsorb for 1h at 37°C in a 0.5% CO_2 controlled incubator. Following adsorption the virus inoculum was removed and the cells were overlaid with 4 ml 50% Agarose (Promega) (1% concentration prepared in Earles Balanced Salt solution), 50% MEM supplemented with 5% fetal calf serum (FCS), 0.5% $CaCO_3$ and antibiotics. The titration plates were incubated at

37°C, 0.5% CO_2 for 5 – 6 days until plaques were clearly visible under light microscopy. The cells were then stained with 0.01% neutral red (Sigma), 1% Agarose (Promega) (prepared in Earles Balanced Salt solution) and incubated overnight. Opaque plaques observed at the various dilutions were counted.

Single virus populations were obtained after triplicate cycles of single plaque purifications following AHSV titrations. Picked plaques were amplified on 25 cm^2 flasks seeded with Vero cells at 50% confluency. When the infection had reached a CPE of 3, the infections were transferred to a 50 ml Sterillin tube and centrifugated at 2000 x g for 10 min in a Beckman J2 – 21 centrifuge at 4°C. The supernatant was transferred to sterile screw cap storage tubes as reserve virus stocks. To monitor mutations introduced through plaque purifications, total RNA extractions were made from the collected cell debri pellets using TRIZOL™ reagent (Gibco BRL) according to manufacturers instructions. The S10 gene segment was amplified from the total RNA isolations using the RT-PCR procedure previously described (see 3.2.2) and then directly sequenced (see 3.2.3). This enabled monitoring any mutations introduced in the plaque selection and purification.

4.2.2 Reverse transcriptase polymerase chain reaction

The S10 gene segments of the AHSV-2 isolates (S2 vaccine and 82/61) were amplified using the RT-PCR procedure previously described (see 3.2.3). The primers annealed to the terminal regions of the S10 gene and incorporated restriction enzyme sites to facilitate directional cloning. The forward primer NS3pBam (5' cgg gat ccg ttt aaa tta tcc ctt g) had a *BamHI* restriction site incorporated (underlined) while the reverse primer, NS3pEco (5' cgg aat tcg taa gtc gtt atc ccg g), included the *EcoRI* restriction site (underlined). A high fidelity Taq polymerase (TaKaRa Ex Taq, TaKaRa) that has 5' - 3' and 3'- 5' exonuclease activity was used to reduce the possibility of cloning mutated reproductions of the S10 genes.

4.2.3 Cloning of S10 amplicons into pFastBac and screening recombinants

The S10 PCR amplicons were precipitated in 95% absolute ethanol to remove any residual β-mercaptoethanol remaining after cDNA synthesis reactions that inhibited restriction enzyme digestions (see 3.2.2). The precipitated S10 amplicons were subsequently digested with 1 U of *BamHI* and 1 U of *EcoRI* (both Roche Diagnostics) for 4 h at 37°C in restriction enzyme buffer B (Roche Diagnostics). The digested S10 amplicons were electrophoresised on a 1% agarose gel for 35 min at 90 V and purified from the gel slice using the High Pure PCR purification kit (Roche Diagnostics). Briefly, the agarose slice containing the desired S10 DNA was weighed and 300 µl of binding buffer (supplied in the kit) was added per 100 mg agarose gel slice and incubated at for 10 min at 50 – 60°C, vortexing every two to three minutes. Following incubation, 150 µl of isopropanol was added per 100 mg agarose gel slice and vortexed thoroughly. Binding buffer (500 µl / 100 µl PCR product) was then added and

the sample was pipetted into a High Pure filter tube, centrifugated at top speed for 5 sec and washed twice with wash buffer. The DNA was eluted in 40 µl of UHQ.

For cloning, approximately 5 µg of the vector pFastBac was digested with 1 U each of *BamHI* and *EcoRI* in buffer B (Roche Diagnostics) for 2 h at 37°C. Following linearization, the vector was electrophoresed on a 0.8% agarose gel, from which it was excised and purified using GeneClean DNA purification kit (Bio101) according to manufacturer recommendations. Ligation reactions contained 200 ng of linearized, purified pFastBac, 750 ng of purified S10 gene inserts and 1 U of T4 DNA ligase in 1x ligation buffer (Roche Diagnostics) (total 10 µl reaction volume) and were incubated at 16°C overnight. Ligation mixtures were transformed into competent *E. coli* JM105 cells (prepared with the $CaCl_2$ method, Sambrook *et al.* (1989)). Possible recombinants were selected from white, ampicillin resistant colonies indicating β-gal gene inactivation. Screening for recombinant vectors containing the S10 gene inserts was by conventional mini preparations (Birnboim & Doly, 1979). Confirmed recombinant colonies were grown in large scale cultures and used for large scale plasmid isolations using conventional methods described by Sambrook *et al.* (1989).

Recombinant plasmids were purified for sequencing through High Pure Plasmid isolation columns (Roche Diagnostics) and sequenced using the Big Dye terminator cycle sequencing kit (PE Applied Biosystems) according to manufacturer recommendations on the ABI 377 automated sequencing system.

4.2.4 Expression of NS3 in Sf9 cells by means of the BAC-TO-BAC™ baculovirus expression system

4.2.4.a Cells and baculoviruses

Spodoptera frugiperda (Sf9) cells (ATCC CRL-1711) were used as baculovirus host cells for protein expression with the BAC-TO-BAC™ (Gibco BRL) system. Sf9 cells have a doubling time of 18 to 22 h in both monolayer and suspension culture. Cells were maintained on 75 cm^3 monolayers in Graces Insect Medium (Highveld Biological) containing 10% FCS (Highveld Biological) and antibiotics (60 mg/ml each of penicillin G and streptomycin, 150 µg/ml Fungizone, all Highveld Biological). Cells adapted to suspension culture were propagated in the medium as described except that 0.008% Pluronic F-68 (Sigma) was added to protect the cells against possible shearing effects. Suspension cultures were seeded at an initial density of 0.2×10^6 cells/ml and subcultured when they reached 2×10^6 cells/ml. The cells were incubated at 27°C. Cell density was determined by counting a cell sample (50% cell suspension / 50% 0.4% Trypan blue (Sigma) in 1 x PBS) using a hemacytometer.

In most instances Sf9 cells were seeded at a density of 1×10^6 cells per 6-well plate, 0.3×10^6 cells per 24-well plate, 1×10^7 cells per 75 cm^3 flask or 2×10^6 cells per 25 cm^3 flask and left

to attach for 45 min before infection. The cells were infected by removing the seed medium and replacing it with fresh medium to which virus stock was added at the desired MOI. Suspension cultures were infected when they reached a density of 1 x 10^6 cells/ml by collecting the cells at 2000 x g for 10 min and re-suspending in one tenth of the original volume. Infections at the desired MOI were left to adsorb for 1 – 2 h at 27°C. The volume was then increased to that of the original and the cells were replaced to incubate for 2 – 4 days, depending on the desired application.

Baculoviruses are widely used as vectors to express heterologous genes placed under the control of the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV). The BAC-TO-BAC™ Baculovirus expression system (Gibco BRL) uses a site-specific transposition to insert foreign genes into a bacmid (baculovirus shuttle vector) that propagates in *E. coli* DH10BAC™. Recombinant virus DNA isolated from selected colonies is not mixed with parental, non-recombinant virus, eliminating the need for multiple rounds of plaque purifications.

4.2.4.b Transposition and isolation of recombinant bacmid DNA

Recombinant pFastBac donor plasmids containing the S10 gene inserts (see 4.2.2) were used to transform DH10BAC competent cells (prepared with the DMSO method, Sambrook *et al.* (1989) and stored at –70°C in 15% glycerol until use). Briefly approximately 5 ng of plasmid was gently mixed with 100 µl of cells, left on ice for 30 min after which a heat shocked was administered for 90 sec at 42°C. 900 µl of S.O.C. (L-broth containing 250 mM KCl, 10 mM MgCl2 and 20 mM glucose) recovery medium was added to the cells that were then placed into a shake incubator at 37°C for 4 h. 100 µl of the transformation mixture was plated onto LB plates containing kanamycin (50 µg/ml), gentamycin (7 µg/ml), tetracyclin (10 µg/ml), X-Gal (100 µg/ml) and IPTG (40 µg/ml). The plates were inverted and incubated at 37°C for at least 24 h.

True white phenotypic colonies were confirmed by re-streaking onto fresh plates (supplemented as above). A single colony was selected and grown overnight in liquid LB (supplemented with antibiotics as described above) to be used for bacmid DNA isolation. A protocol specifically developed for isolating plasmids larger than 100 kb adapted for isolating bacmid DNA was followed according to manufacturer recommendations (Gibco BRL). All solutions were freshly prepared and filter sterilized prior to use. The procedure is similar to that of a conventional mini-prep except that all reagents are gently added and mixed. The DNA pellet was air dried in a sterile flow laminar and dissolved in 40 µl of sterile 1 x TE buffer by occasionally gently tapping the tubes, being careful to avoid any mechanical shearing.

4.2.4.c Transfection of Sf9 cells with recombinant bacmid DNA

Sf9 cells cultured to a mid-log phase with a 97% viability were seeded at a density of 1 x 10^6 onto 6-well plates in 2 ml of Graces insect media (Highveld Biological) without serum and with half antibiotic concentration and left for 1 – 2 h to attach during which time two solutions were prepared as follows;

Solution A: 10 µl of mini-prep bacmid DNA and 100 µl of Graces medium without serum or antibiotics

Solution B: 6 µl CELLFECTIN Reagent™ (Gibco BRL) mixed thoroughly in 100 µl Graces insect medium.

The two solutions were combined, gently mixed and incubated for 45 min at room temperature.

The attached Sf9 cells were washed twice with 2 ml of Graces insect medium without antibiotics. To each tube containing the lipid DNA mixture 800 µl of Graces insect medium was added, gently mixed and used to overlay the Sf9 cells. The cells were incubated at 27°C for 5 h. After incubation the transfection mixture was removed from the wells and replaced with 2 ml of Graces insect medium supplemented with 10% FCS and antibiotics. The cells were incubated as described above for a further 48 h after which the primary transfection mixture was removed and stored in storage tubes at 4°C.

4.2.4.d Amplification of recombinant baculoviruses

Recombinant primary transfection viruses were amplified to increase the initial virus titres prior to screening for the presence of AHSV-2 NS3. Briefly, 200 µl of primary transfection mixture was used to infect Sf9 cells seeded at 1 x 10^6 cells per 6-well in a total volume of 1 ml Graces insect medium supplemented with FCS and antibiotics as described. The plates were placed in an aseptic sealed container with a bit of dampened paper to prevent medium evaporation at 27°C for 72 h. This cycle was repeated once again using the secondary transfection mixture and the infected cells from this infection were screened for NS3 expression.

4.2.4.e Baculovirus titrations

Baculoviruses were titrated essentially as described by O'Reilly *et al.* (1992) but with minor modifications. Briefly 6-well plates were seeded with cells at a confluency of 2x10^6 cells per plate and incubated at 27°C for 1 h. During this time the baculovirus titrations were prepared in the range of 10^{-2} to 10^{-7} in Graces medium without supplements (Highveld Biological). The attached cells were infected with 1 ml of the respective titrations (in duplicate) and left to incubate for 2 h at 27°C with gentle rocking. A 6% low melting agarose (Sigma) solution, prepared in ddH_2O, was autoclaved for 15 min and kept as a sterile agarose stock. A working solution was diluted to a concentration of 3% in Graces medium (Highveld Biological) that had been pre-heated to 37°C. Following absorption, the virus titrations were gently aspirated from

the cells and replaced with 4 ml of the prepared agarose overlay. The plates were left at room temperature until the agarose had solidified and then incubated at 27°C for 3 to 4 days. The cells were monitored every day for signs of plaques. Once plaques were clearly visible the cells were stained with 1 ml of a 100 µg/ml neutral red solution prepared in 1% agarose (made up in ddH_2O and autoclaved for 15 min). The plates were incubated overnight as described above and opaque plaques were counted the following day.

4.2.5 [^{35}S]-Methionine pulse labeling of Sf9 cells

Sf9 cells were seeded at 1 x 10^6 cells per 6-well and left to attach for 1 h at room temperature. The cells were infected with different volumes of tertiary transfection mixture (50, 100, 250 and 500 µl respectively) while one well was left uninfected (mock infected) and the last well of the 6-well plate was infected with a wild type non-recombinant baculovirus to confirm successful infection. After 1 h the infection mixtures were removed and replaced with 2 ml of supplemented Graces insect medium and incubated for a further 18 to 20 h at 27°C. The infected Sf9 cells were washed twice with Eagles medium without methionine (Highveld Biological) taking care not to disturb the Sf9 cell monolayer. The infected cells were starved of methionine in 1 ml of fresh wash medium as described above and incubated for a further 1 h at 27°C. Following incubation the medium was replaced with 1 ml of Eagles without methionine containing 30 µCi [^{35}S]-methionine (ICN Pharmaceuticals) /ml and the plates were returned to incubate for a further 3 to 4 h. The cells were harvested from the medium by centrifugating the entire cell sample collected in the labeling medium for 4 min at 500 x g in a benchtop centrifuge. Protein expression was analysed by electophoresis of the lysed cell pellet (equal volume 1 x PBS, 2 x PSB) on a 12% SDS polyacrylamide gel using standard electrophoresis conditions (120 V for 1.5 h). The gels were dried and exposed to X-ray films in a cassette for approximately 48 h after which the exposed films were developed and fixed according to standard autoradiographic methods to visualize all labeled cellular proteins.

4.2.6 Production of α-AHSV-2 NS3 serum

4.2.6.a Cloning the S10 gene into the bacterial expression vector pUEX-3

The pUEX (Amersham Life Sciences) bacterial expression vectors express the foreign inserted protein as a carboxyl terminal fusion product of the β-gal protein (Bressan & Stanley, 1987). β-Gal expression is induced by a heat shock treatment of 42°C and high yields of the protein are rapidly obtained in transformed *E. coli* cell lines such as JM105 or XL1 Blue cells. The pUEX-3 (gift from Department of Biochemistry, O.V.I.) vector provided the correct reading frame for expressing AHSV-2 NS3 from the inserted S10 gene.

The AHSV-2 vaccine S10 gene was recovered from the recombinant pFastBac vector (see 4.2.3) by digestion with *BamHI* and *PstI*. The S10 fragment was purified from the digestion

mixture by excising the corresponding S10 band from a 1% agarose gel following electrophoresis and purifying the DNA from the slice with an adapted protocol for gel purification with the High pure PCR purification kit (Roche Diagnostics) (see 4.2.3)

Cells harboring the pUEX vectors were always grown at 30°C and transformations were performed at a heat shock temperature of 34°C instead of 42°C (Bressan & Stanley, 1987). The pUEX-3 vector was digested with the same restriction enzymes used to recover the S10 gene from pFastBac. This forced directional cloning of the S10 gene. The linearized vector was purified as previously described for the S10 insert and 200 ng was ligated in the presence of 700 ng S10 gene insert as previously described (see 4.2.3). The ligation mixture was transfomed into JM105 cells and possible recombinant colonies (Amp^r) were grown overnight at 30°C to be screened by conventional mini-preparations as previously described (Doly, 1978). Recombinant plasmids were confirmed by digesting the plasmids with *PstI* and *BamHI* (both Roche Diagnostics) for 2 h at 37°C.

4.2.6.b Expression of β-galactosidase NS3 fusion protein

JM105 colonies that harbored a copy of the recombinant pUEX-AHSV-2 S10 plasmid were re-streaked onto fresh plates containing ampicilin and incubated overnight at 30°C. A single colony was selected and used to inoculate an overnight broth culture incubated at 30°C with agitation. The overnight culture was diluted the following morning 1:10 and incubated as above until cells were in the logarythmic phase. Expression of the β-gal NS3 fusion protein was induced by transferring the cultures to a 42°C shake incubator for a further 2 h. Aliquots of 100 µl of the uninduced cell samples were removed prior to the temperature change to serve as uninduced controls.

To screen for fusion protein expression, 200 µl aliquots were removed from the induced cultures and the bacterial cells were collected in a bench top centifuge at top speed for 4 min. The collected cells were prepared for SDS-PAGE by re-suspending the cells in 20 µl of 1 x PBS to which 40 µl of 2 x PSB was added. The protein samples were boiled for 5 min and loaded onto a 10% polyacrylamide gel. Electrophoresis was done at 130 V for 2 h. Following electrophoresis the gels were stained with 0.05% Coomasie blue for 10 min and destained in a 5% methanol / 5% acetic acid solution overnight.

4.2.6.c Fusion protein purification

Overnight cultures were inoculated for large scale β-gal NS3 fusion protein production as described above but scaled up to 250 ml. The bacterial cells were pelleted at 7000 x g with a Beckman J2–21 centrifuge at 4°C for 10 min. The bacterial cells were lysed in 2 ml of lysis buffer (1 mM Tris-HCl, pH 8.0, 0.4 mM EDTA, 20% sucrose w/v, 5 mg/ml lysozyme). The soluble and insoluble protein fractions were separated at top speed for 5 min in a bench top IEC Micromax centrifuge. Both fractions were analyzed by electrophoresis. Large (25 cm x

25 cm) 10% polyacrylamide gels were prepared and the protein samples were prepared for elecrophoresis as previously described (see 4.2.6.b). Cell fraction associated protein samples were electrophoresed overnight at 100 V in the Model SE400 Vertical Slab Gel Units (Hoefer Scientific Instruments). The gels were not stained and the location of the β-gal NS3 fusion protein band was estimated by comparison to the molecular weight rainbow marker and a gel electrophoresed under identical conditions that had been stained and destained (see 4.2.6.b). The region corresponding to the β-gal NS3 fusion protein was excised with a sharp scalpel blade and placed into a clean, sterile corex tube that had been siliconized. The gel slice was Ultra-Turrax homogenized in 1 ml elution buffer (50 mM Tris pH 9.0, 1% Triton X-100, 2% SDS) and incubated at 37°C with shaking for 2 h. The acrylamide was removed by leaving the homogenized mixture at 4°C overnight or alternatively by centrifugation in the J2-21 Beckman centrifuge at 3000 x g for 15 min. The purified protein was precipitated from the supernatant by addition of 4 volumes ice-cold acetone and collected after 1 h at –70°C by centrifugation at 18 000 rpm for 30 min in a SW28 rotor using a L–70 Ultracentifuge (Beckman). The protein pellet was dried and re-suspended in a final volume of 600 µl filter sterilized 1 x PBS. The purified protein sample was electrophoresed on a mini 10% polyacrylamide gel to verify purification and estimate protein concentration.

4.2.6.d Immunization schedule

A rabbit was the animal of choice for the production of the serum directed against the β-gal NS3 fusion protein as it is easy to handle and could provide sufficient quantities of serum. The rabbit was kept at O.V.I were it was injected and bled for serum. The immunization schedule is given in Table 4.1.

Table 4.1 Immunization schedule for the production of the AHSV-2 NS3 serum

Date	Antigen concentration	Adjuvant	Route of administration
26/05/2000*	37 µg in 250 µl 1 x PBS	250 µl ISA 50 (Montanida, France)	I.M.
	37 µg in 500 µl 1 x PBS	1 mg Quilaja saponin	I.M.
05/07/2000* (day 40)	25 µg	250 µl ISA 50	I.M.
	25 µg	1 mg Quilaja saponin	I.M.
12/07/2000 (day 47)	Bleed: 10 ml from lateral ear vein		
03/08/2000 (day 69)	25 µg	250 µl ISA 50	I.M.
	25 µg	1 mg Quilaja saponin	I.M.
10/08/2000 (day 76)	Bleed: 25 ml from lateral ear vein		

* Antigen was injected into a single rabbit in two separate shots with specified adjuvants

4.2.7 Western blot analysis

Following PAGE, the separated protein samples were transferred from the polyacrylamide gel onto a nitrocellulose membrane (Hybond-C extra, Amersham Life Sciences) in a submerged EC 140 mini blot module (E-C Apparatus Corporation) with 1 x Towbin buffer without methanol (25 mM Tris, 192 mM Glycine) as transfer buffer. Following protein transfer at 16 V (0.1 mA) for 45 min, the membranes were rinsed in 1 x PBS for 5 min with agitation at room temperature and then blocked for 30 min in 1% blocking buffer (1% low fat milk powder in 1 x PBS) with agitation at room temperature. The blocked membranes were placed into a plastic bag to which 20 ml of primary antibody was added (1:100 dilution of rabbit serum in blocking buffer) and left to incubate overnight at room temperature with gentle agitation. The membranes were removed from the sealed bags the following morning, placed in a suitable open container and washed three times with 1 x washing buffer (0.05% Tween in 1 x PBS) for 5 min each time to remove non-specific antibody bound complexes. The membranes were transferred to a new bag and 20 ml of secondary antibody was added (1:250 Peroxidase-conjugated Protein A in 1 x blocking buffer, ICN Pharmaceuticals) and left to incubate as described for a further hour. The wash procedure described above was repeated and a final

wash for 5 min in 1 x PBS was included. For colormetric detection a fresh enzyme substrate was prepared (60 mg 4-chloro-1-naphtol (Sigma) in 20 ml ice-cold methanol added to 60 µl of H_2O_2 in 100 ml of 1 x PBS) in which the membrane was placed until bands became visible. The membranes were then rinsed in H_2O and left to dry.

4.2.8. $[^{45}Ca]^{2+}$ Influx assays

Membrane permeability of infected Sf9 cells was assessed by quantitating the influx of radiolabeled $^{45}[Ca]^{2+}$ into cells (Miller *et al.*, 1993). Spin cultures of Sf9 cells were infected with different recombinant baculoviruses at a MOI of 5 pfu/cell. At different times p.i. samples were removed and the cells counted using a hemacytometer to determine the number of cells per ml to standardize the size of the cell sample (3 x 10^6 cells) for the influx assays. The correct number of cells was pipetted into a clean 1.5 ml eppendorf tube and 10 µCi of $^{45}[Ca]^{2+}$ was added to the cells on ice. To assay the permeability of membranes, a 20 µl aliquot was removed from the suspension every minute, over a 4 min period, following the addition of $^{45}[Ca]^{2+}$ and the cells were centrifuged at top speed in a IEC Micromax bench top centrifuge for 20 sec to pellet the cells. The cells were rinsed with 200 µl ice cold 1 x PBS without disturbing the cell pellet to remove excess $^{45}[Ca]^{2+}$, briefly centrifugated at top speed again and the excess 1 x PBS was removed from each tube. The tube containing the labeled cell pellet was placed directly into a scintillation vial to which scintillation fluid was added. The sample was soaked overnight and the $^{45}[Ca]^{2+}$ influx into the cells was measured with a Beckman 3801 β counter. As a control for maximum permeability, 1 µl of 200 µM digitonin was added to the last aliquot of cell suspension and allowed to incubate for 1 min. Digitonin permeabilizes the plasma membrane of eukaryotic cells by complexing with membrane cholesterol and other unconjugated β-hydroxysterols. This value (CPM) was used to determine the maximum index of $^{45}[Ca]^{2+}$ influx at a given time and was used to calculate the percentage $^{45}[Ca]^{2+}$ influx for each of the infections. The influx of $^{45}[Ca]^{2+}$ was assayed as described above.

4.2.9 AHSV release assays from Vero cells and associated cytopathic effect

For the AHSV release assays Vero cells were seeded at 50% confluency on 6-well plates. The cells were infected approximately 18 h later with each of the virus isolates at a MOI of 5 in 1 ml of unsupplemented MEM for 45 min. After adsorption, the virus innoculum was removed from each plate and replaced with 1 ml of fresh MEM supplemented with 5% FCS and antibiotics. The plates were incubated at 37°C with 0.5% CO_2 for time intervals of 24, 48 and 72 h. A day prior to harvesting the infected cells and cell supernatants, 6-well plates were seeded with Vero cells at 60% confluency to be used immediately once dilutions of the cell pellet and supernatant fractions were prepared. Cells were harvested with a cell scraper and collected in the MEM present in each well (approximately 1 ml) which was centrifugated at 4000 x g for 10 min to separate the cellular fraction from the cell supernatant fraction. The exact volume of the supernatant was withdrawn and noted and the cell pellet was

resuspended in 1 x PBS to yield the same volume. The cell pellet fraction was subsequently passed 10 times through a 22 G needle to mechanically break the cells open and release virus particles associated with the cells. A 200 µl aliquot of each of the cell supernatant and cell pellet fractions was titrated to a dilution of 10^{-6} and 1 ml aliquots of each dilution was used to infect cells on 6-well plates prepared the day before. The titrations were done as previously described (see 4.2.1.).

4.2.10 Hygromycin B membrane permeabilization assays of AHSV infected Vero cells

Vero cells were seeded on 24-well plates at 50% confluency and incubated overnight at 37°C with 0.5% CO_2 in MEM growth medium supplemented with 5% FCS and antibiotics. Three wells were seeded for each time post infection at which the assay was to be done for each virus isolate included. The growth medium was removed the following morning and the cells were infected with the virus stocks at an MOI of 3 in 1 ml of supplemented growth medium. The membrane permeabilization assay was based on a method described by Chang *et al.* (1999). At indicated times p.i. (6, 12, 18 or 24 h) the medium was removed from the infected cells and the cells were washed twice with MEM without methionine. The medium was replaced with MEM without methionine on two of the infected wells. The third infected well was kept as a control for an immunoblot to screen for NS3 expression in the cells. Hygromycin B (500 µg/µl, Roche Diagnostics) was added to one of the two wells that contained MEM without methionine. The cells were left to incubate for a further 30 min. The medium was removed from the wells and replaced with MEM without methionine and 100 µCi [^{35}S]-methionine (ICN Pharmaceuticals) and incubated for a labeling period of 30 min. The medium was removed from the labeled cells and the cells were rinsed twice in 1 x PBS to wash excess labeling medium from the cells. The cells then were harvested in 50 µl of lysis buffer (1% Nonidet P-40; 150 mM NaCl; 50 mM Tris-HCl pH 7.5, 1 mM EDTA with 20 µg/ml of phenylmethylsulfonyl flouride added just before use) to which 50 µl of TCA solution (5% trichloroaecetic acid; 20 mM sodium pyrophosphate) was immediately added to prepicitate proteins. A 50 µl aliquot of each sample was spotted onto fibreglass discs (GF/C, Whatman). The discs were washed in 70% ethanol and left to dry at room temperature. The discs were placed in scintillation fluid (Beckman) and counted in a Beckman 3801 β counter. The percentage [^{35}S]-methionine incorporated into the permeabilized cells was calculated as follows:

$$\text{CPM of sample containing Hygromycin B} \;/\; \text{CPM of sample in absence of Hygromycin B} \times 100$$

The index values were used to plot [^{35}S]-methionine incorporation graphs.

4.3 RESULTS

4.3.1 Characterization of AHSV-2 vaccine strain and AHSV-2 82/61 reference strain S10 genes and encoding NS3 proteins

As previously established (Chapter 3) the NS3 sequence of the vaccine strain (S2VAC) differed from the original 82/61 reference strain (S2REF) by four amino acids. The specific amino acid substitutions were S to G at position 9, R to Q at position 91, Q to P at position 95 and Y to H at position 117 as seen in the AHSV NS3 sequence alignment (Fig 3.1). In order to identify any possible differences between the NS3 proteins of the AHSV-2 vaccine and reference strains, the S10 genes for each of the virus isolates were cloned and expressed.

4.3.1.a Plaque purification of AHSV-2 82/61 and S10 gene sequence confirmation

The original AHSV-2 82/61 isolation was made in 1961 from a horse that had died from AHS and the strain was thus considered to be virulent. This virus, selected as the AHSV-2 reference strain 82/61, had been lyophilized and stored in liquid N_2 as a fourth generation mouse brain passage at the Orbivirus Reference Laboratory, O.V.I. The virus had not been plaque purified and possibly consisted of various sub-populations. It was therefor necessary to obtain a plaque purified strain of AHSV-2 82/61 to ensure that the same virus was to used throughout the series of planned experiments.

To obtain a pure isolate of the reference strain 82/61 the mouse brain passaged stock was plaque purified through three cycles of plaque purifications. To ensure that there was no bias selection for a virus that differed in NS3 sequence from the original 82/61 strain, three random plaques were selected after every purification, amplified in Vero cells and the S10 dsRNA extracted for reverse transcription PCR and direct sequencing. The 82/61 S10 gene sequences were in all instances, except one, identical to that of the first sequences obtained after the first plaque purification cycle. One of

the plaques selected after the second cycle of amplification had a change in position 331 of the S10 gene (cytosine to thymine). This mutation however did not alter the amino acid codon, serine, and was therefor only a silent mutation. The 82/61 S10 gene with cytosine at position 331 was used in all the subsequent experiments

4.3.1.b Cloning AHSV-2 S10 amplicons into pFastBac

S10 genes of the AHSV-2 vaccine and virulent 82/61 strains were amplified by RT-PCR with primers containing restriction enzyme sites. The amplicons were cloned into the *BamHI* and *EcoRI* sites of the bacmid donor plasmid, pFastBac (see 4.2.3). Recombinant pFastBac plasmids from which S10 inserts could be recovered by *BamHI* and *EcoRI* restriction enzyme digestions were obtained (Fig 4.1). The recombinant plasmids were labeled pFB-AHSV-2 Vac-S10 and pFB-AHSV-2 82/61-S10 respectively. To verify the sequences, the plasmids were sequenced (see 4.2.3) using a primer (5' tta att aaa ata cta tac tgt) designed to anneal in the p10 polihedrin region of pFastBac as well as the S10 reverse primer, NS3pEco (see 3.2.3). The sequence obtained confirmed that the genes were cloned in the correct orientation and that the sequences of the cloned S10 genes were identical to those of the directly sequenced S10 PCR amplicons previously determined (see 3.2.3). These recombinant plasmids contained the authentic AHSV-2 S10 gene inserts in the correct orientation for transposition into bacmid DNA.

4.3.1.c Expression of AHSV-2 NS3 proteins using the BAC-TO-BAC™ baculovirus expression system

To compare the cytotoxic effect of the AHSV-2 vaccine and virulent NS3 proteins in infected cells it was necessary to express these proteins in insect cells by means of the corresponding baculovirus recombinants. The AHSV-2 S10 genes cloned into pFastBac were transposed into bacmid DNA (see 4.2.4.b). Recombinant baculovirus stocks were produced following the transfection of the isolated recombinant bacmid DNA into Sf9 cells (see 4.2.1.c). These stocks (BAC-AHSV-2 Vac-NS3 and BAC-AHSV-2 82/61-

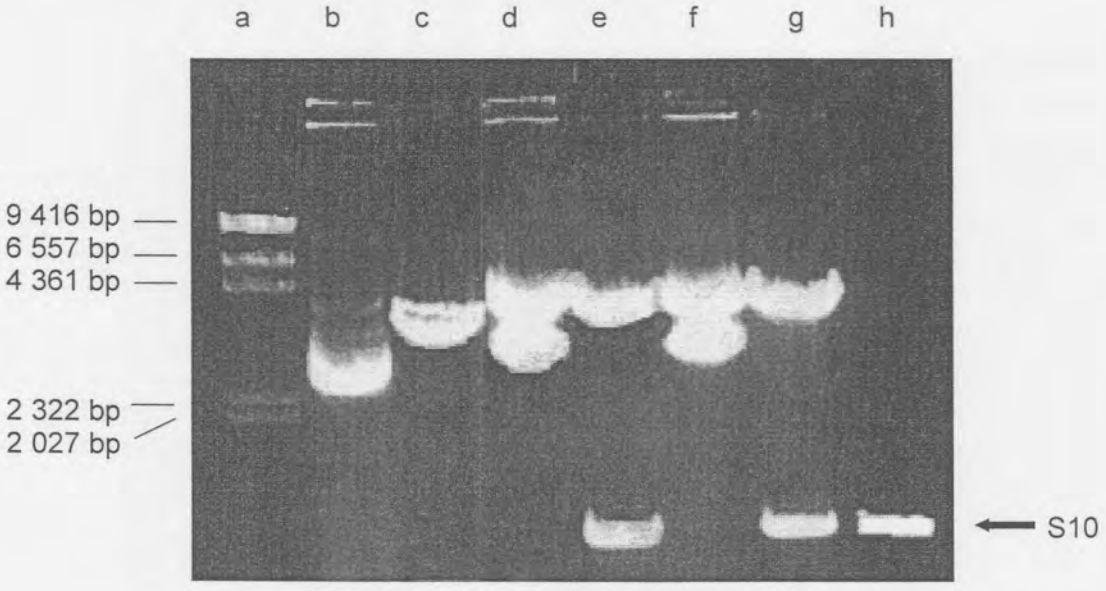


Fig 4.1 Agarose gel showing pFastBac recombinants. Plasmids pFastBac (b), pFB-AHSV-2 Vac-S10 (d) and pFB-AHSV-2 82/61-S10 (f). pFB-AHSV-2 Vac-S10 was digested with *EcoRI* (c) and pFB-AHSV-2 Vac-S10 (e) and pFB-AHSV-2 82/61-S10 (g) were digested with *BamHI* and *EcoRI*. Molecular weight marker II (a) and S10 RT-PCR amplicon (h) were included as size markers. The arrow indicates the AHSV S10 insert.

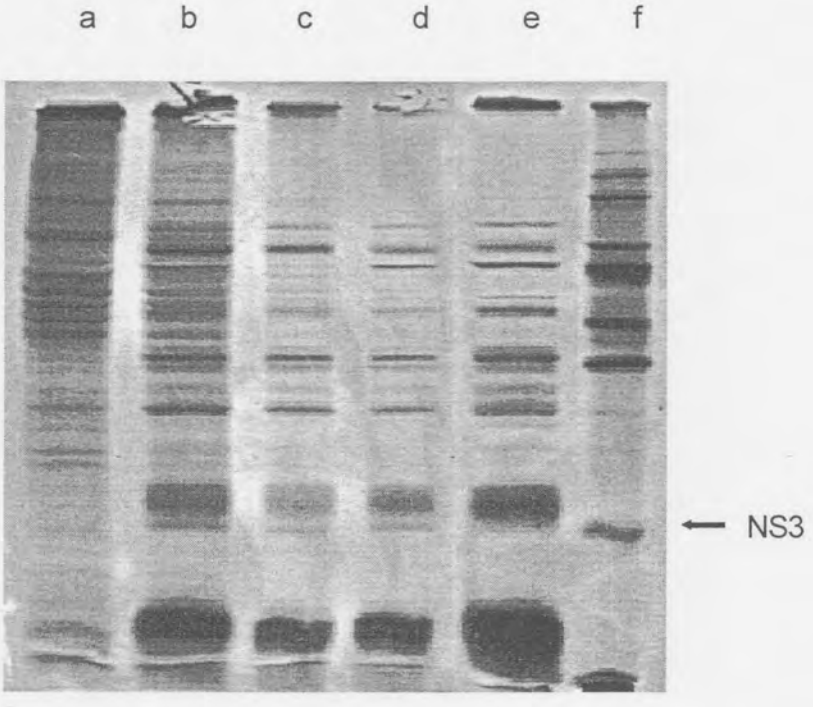


Fig 4.2 Baculovirus expression of AHSV-2 NS3. SDS-PAGE separation and autoradiography of pulse labelled Sf9 cell extracts of mock infected (a), BAC-AHSV-2 Vac-NS3 infected at an MOI of 1 (b) and MOI of 3 (c), BAC-AHSV-2 82/61-NS3 infected at an MOI of 3 (d), wild type infected baculovirus cells (e) and *in vitro* translated dsRNA of AHSV-2 vaccine as control (f).

NS3), once amplified, were used to infect cells to express the AHSV-2 NS3 proteins. The level of AHSV-2 NS3 expression in Sf9 cells was however insufficient to detect the protein by means of conventional Coomassie staining methods and a Western blot with primary antibodies elicited against AHSV-3 NS3 (Van Staden, 1993) failed to detect AHSV-2 NS3 proteins (results not shown). Expression of AHSV-2 NS3 proteins was subsequently confirmed by [^{35}S]-methionine pulse labeling of infected cells at 18 to 20 h p.i. and analysis by 12% SDS-PAGE and autoradiography (Fig 4.2) The presence of an unique protein band, not seen in wild type infected cells of approximately 24 kD in size, was observed in extracts of cells expressing AHSV-2 vaccine and AHSV-2 82/61 NS3 recombinants(Fig 4.2, lanes b, c and d). As a control, purified AHSV-2 vaccine strain dsRNA was *in vitro* translated (see 3.2.4), (Fig 4.2, lane f). The *in vitro* translated S10 gene segment corresponded with the position of the unique 24 kD protein band of the pulse labeled infections thereby confirming NS3 expression.

Expression of AHSV-2 vaccine and virulent NS3 protein from recombinant baculoviruses in Sf9 cells allows for the comparison of the membrane damaging potential induced by these two proteins. In order for such assays to be conclusive, it would be necessary to confirm AHSV-2 NS3 expression at different stages by e.g. immunoblot. This would require antibodies specific to AHSV-2 NS3.

4.3.2 Production of α-AHSV-2 NS3 serum

Monospecific serum directed against AHSV-3 NS3 failed to detect AHSV-2 NS3 in infected Sf9 cell extracts and in Vero cells infected with AHSV-2 (Van Staden, 1993). This is probably due to the large amount of variation between AHSV-2 and AHSV-3 NS3 sequences (see Chapter 3).

4.3.2.a Cloning AHSV-2 vaccine strain S10 gene into pUEX-3

In order to obtain large quantities of AHSV-2 NS3 for immunization purposes, AHSV-2 vaccine NS3 was expressed as an inducible β-gal fusion protein using the pUEX-3 vector. The AHSV-2 vaccine S10 gene fragment was recovered from pFB-AHSV-2 Vac-S10 following *BamHI* and *PstI* restriction enzyme digestions, purified and cloned into the pUEX-3 bacterial expression vector using the same restriction enzyme sites (see 4.2.6.a). The results are shown in Fig 4.3. Recombinant pUEX-AHSV-2 S10 plasmids from which the S10 gene was successfully excised by digestion with *BamHI* and *EcoRI* (Fig 4.3, lanes e, f and g) were obtained. Cells harboring the recombinant pUEX-AHSV-2 S10 vectors were subsequently induced to express AHSV-2.

4.3.2.b Expression and purification of the β-gal NS3 fusion protein

Overnight cultures of *E. coli* JM105 colonies harboring pUEX-AHSV-2 S10 were diluted and induced to screen for β-gal NS3 expression. A single transformed culture that showed strong expression was selected and induced for large-scale purification of the fusion protein. A 10% SDS-polyacrylamide gel was prepared for electrophoresis to enhance separation of the large fusion protein. Protein profiles of the cell extracts following electrophoresis (Fig 4.4) indicated that the induced non-recombinant pUEX-3 transformants expressed wild type β-gal (Fig 4.4, lane b) while the induced recombinant pUEX-AHSV-2 S10 transformants expressed the β-gal NS3 fusion protein (Fig 4.4, lane c). The larger size of the fusion protein (130 kD) compared to that of wild type β-gal (116 kD) was as a result of the additional NS3 amino acid residues.

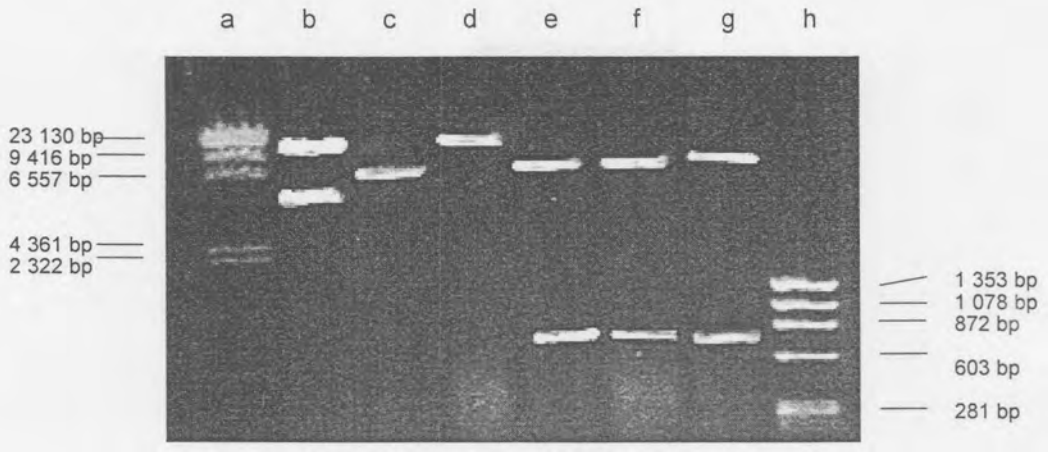


Fig 4.3 Agarose gel showing pUEX-3 recombinants. Undigested pUEX-3 (b), *BamHI* digested pUEX-3 (c), recombinant pUEX-AHSV-2 S10 digested with *BamHI* (d), and pUEX-AHSV-2 S10 recombinants digested with *BamHI* and *EcoRI* (e, f and g). Molecular weight marker II (a) and Phi X lamba (h) were included as size markers.

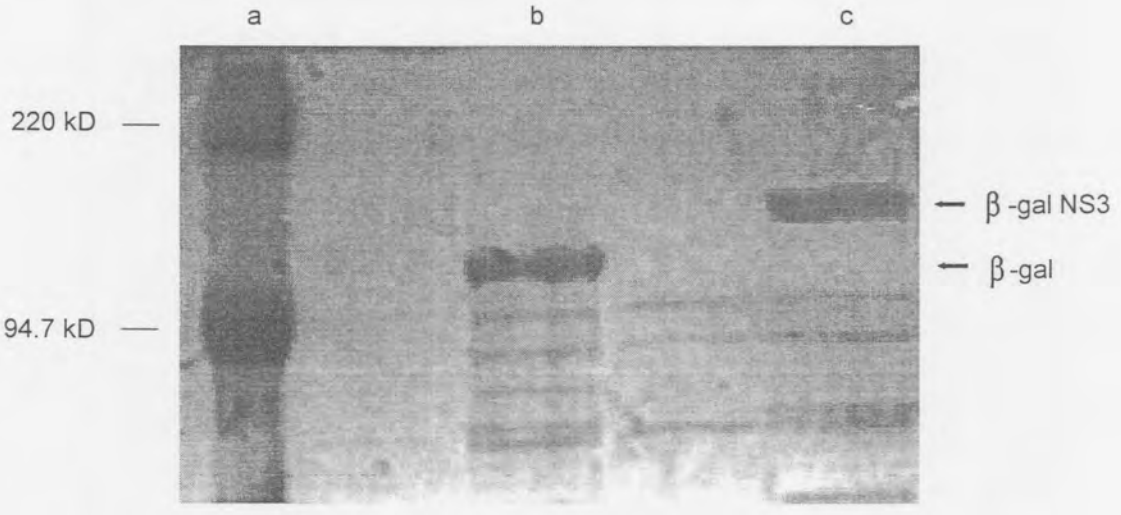


Fig 4.4 Expression of AHSV-2 vaccine NS3 as a β-gal fusion protein. Protein samples were analysed by 10% SDS-PAGE. Rainbow size marker (a), induced JM105 cells harboring wild type pUEX-3 expressing β-gal (116 kD) (b) and pUEX-3 AHSV-2 S10 expressing β-gal NS3 (130 kD) (c).

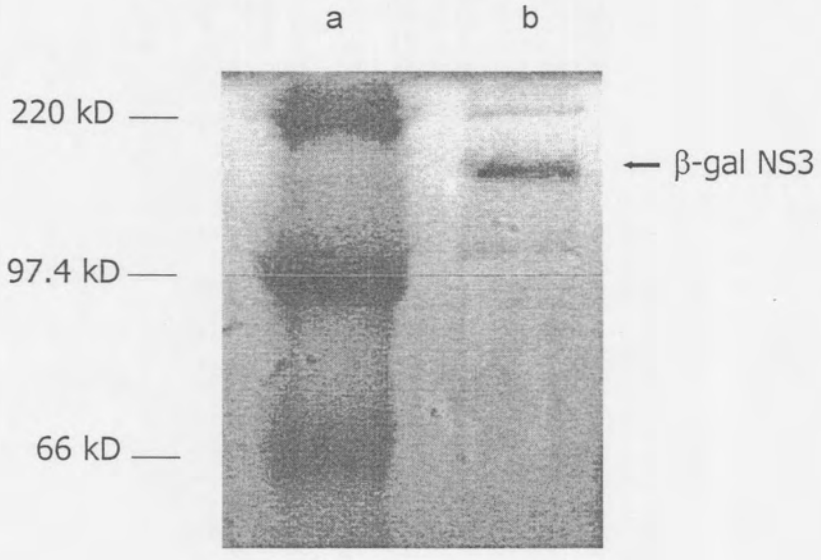


Fig 4.5 Analysis of the purified β-gal fusion protein. Purified β-gal NS3 (b) and rainbow protein marker (a) were compared by 10% SDS-PAGE.

To minimize an immune response to unwanted bacterial proteins, the β-gal NS3 fusion protein expressed after induction of the bacterial cells was recovered from the polyacrylamide gel fragment following electrophoresis (see 4.2.6.b). Following purification (see 4.2.6.c), the protein sample was analysed on a 10% polyacrylamide gel to evaluate protein purity and estimate the final concentration (Fig 4.5). The purified protein concentration was estimated to be 0.5 μg/μl. Sufficient protein (about 300 μg) had therefor been produced for the primary immunization and subsequent boosts necessary to elicit an immune response in the rabbit against AHSV-2 NS3.

4.3.2.c Production of α- β-gal NS3 antibodies

The purified β-gal NS3 fusion protein was used as antigen to produce α-AHSV-2 NS3 serum in a rabbit. To ensure that the selected rabbit would provide serum with low background levels, a pre-bleed serum sample was diluted 1:50 in 1% blocking buffer and used as primary antibody in a Western blot analysis with Sf9 protein samples as antigen. The pre-bleed serum had no response to the Sf9 proteins and the rabbit was therefor suitable for β-gal NS3 immunizations. The primary β-gal NS3 fusion protein exposure and first boost was done by dividing the total protein sample to be administered (i.e. 74 μg for the initial exposure and 50 μg for the first boost) into two equal portions and coupling these samples with two different adjuvants namely ISA (Incomplete Seppic Adjuvant) 50 and Quilaja saponin. This strategy had previously been found to enhance the immune response process (M. Romito, personal communication).

Serum obtained from the first bleed and second bleed tested negative for the presence of α-AHSV-2 NS3 antibodies in a Western blot analysis. A second booster immunization was administered to the rabbit and the resultant serum was again screened for the presence of α-AHSV-2 NS3 antibodies. Protein samples of Sf9 cell extracts expressing AHSV-2 vaccine NS3, AHSV-2 82/61

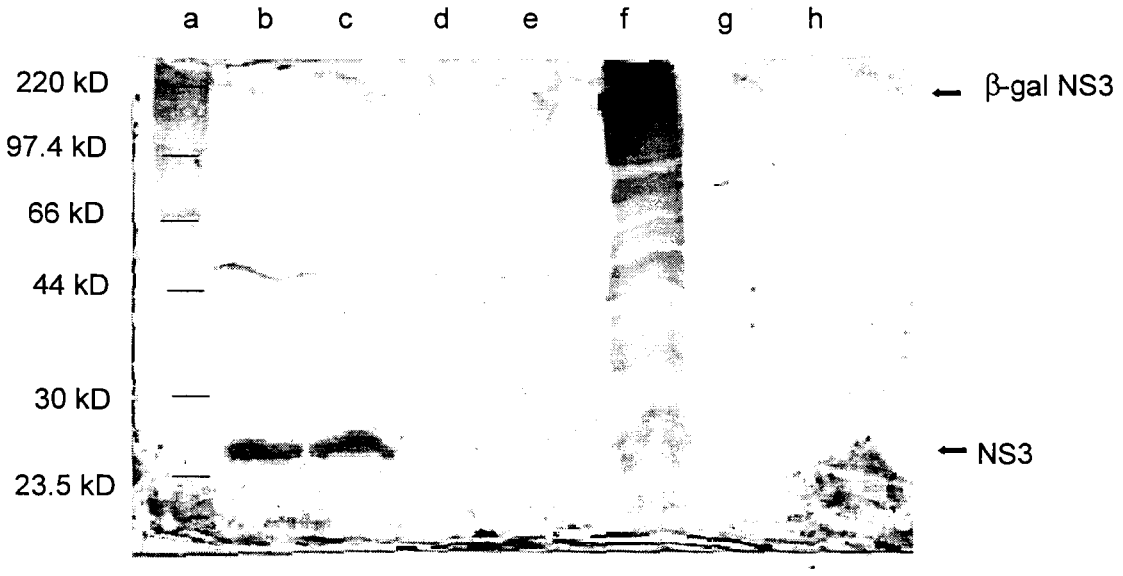


Fig 4.6 Western Blot testing the specificty of α-AHSV-2 NS3 serum. Cell exracts of Sf9 cells expressing AHSV-2 Vac-NS3 (b), AHSV-2 82/61-NS3 (c), AHSV-3 NS3 (g) and AHSV-6 NS3 (h), uninfected Sf9 cell extract (d), wild type baculovirus infected cell extract (e) and β-gal NS3 expressed in *E. coli* cell extracts (f) were prepared by 12% SDS-PAGE and reacted with serum produced from an AHSV-2 β-gal NS3 fusion protein. Rainbow protein size marker (a) was included as a size control.

NS3, AHSV-3 NS3 (Stoltz, 1994) and AHSV-6 NS3 (Smit, 1999) from recombinant baculoviruses with the β-gal AHSV-2 NS3 (positive control) fusion protein and wild type infected Sf9 cell extract (negative control) were separated using standard 12% SDS-PAGE procedures. The proteins were transferred onto a membrane and tested against the serum obtained after the second boost (Fig 4.6). This serum sample (1:100 dilution) recognized AHSV-2 NS3 expressed in Sf9 cells on the membrane (lanes b and c) and therefor contained AHSV-2 NS3 specific antibodies. A strong antibody response to the β-gal NS3 fusion protein was also observed (Fig 4.6, lane f). Numerous smaller proteins were also detected indicating that the purified NS3 was still contaminated with other bacterial proteins or that the extract contained several degradation products. The serum showed enough specificity however not to bind to any Sf9 cellular or wild type baculovirus proteins (Fig 4.6, lanes d and e). The α-AHSV-2 NS3 serum did not cross react with AHSV-3 NS3 or AHSV-6 NS3 (Fig 4.6, lanes g and h respectively). The serum could therefor be used to confirm the expression of AHSV-2 NS3 protein in infected cell extracts and could further differentiate the γ NS3 proteins from α and β NS3 proteins.

4.3.3 Membrane permeability changes

It was of interest to investigate whether there are any differences between membrane permeabilization of the AHSV-2 vaccine and AHSV-2 82/61 NS3 proteins. These experiments could indicate if the four amino acid differences between the proteins affect how NS3 interacts with the membrane. The proteins were expressed in Sf9 cells using the BAC-TO-BAC™ system.

To assess changes in permeability of infected cell membranes, the influx of radiolabeled Ca^{2+} into infected Sf9 cells was monitored. Briefly, $[^{45}Ca]^{2+}$ was added exogenously to mock infected Sf9 cells or Sf9 cells infected with wild type baculovirus or recombinant baculovirus expressing the two different AHSV-2 NS3 proteins. The influx was measured at different times p.i. over a

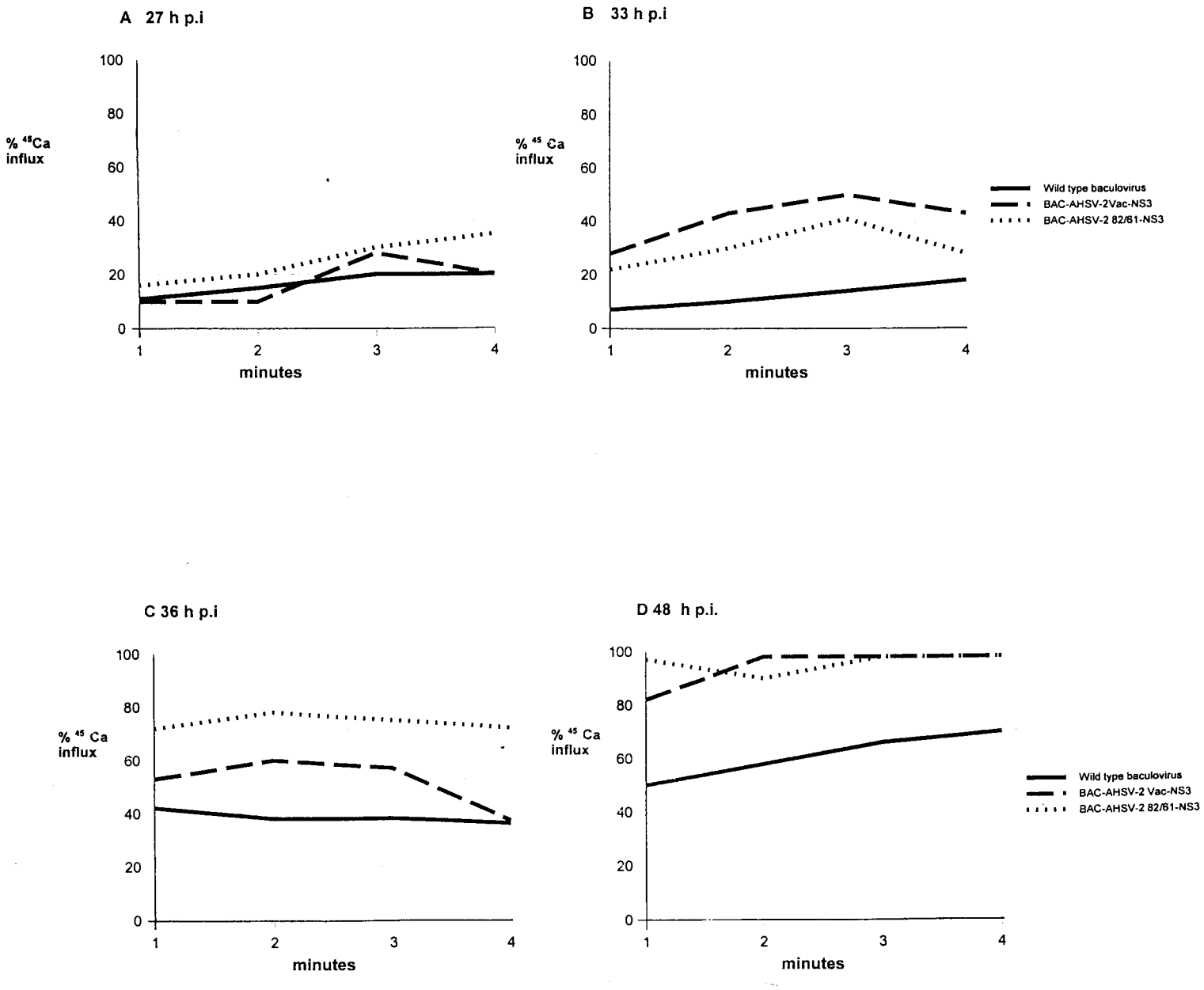


Fig 4.7 Membrane permeabilization measured by $[^{45}Ca]^{2+}$ influx into Sf9 cells infected with either wild type baculovirus, BAC-AHSV-2 VAC-NS3 or BAC-AHSV-2 82/61-NS3 baculovirus recombinants.

period of 4 min (see 4.2.8) and the radioactivity associated with the cell sample was counted (Fig 4.7). Calcium ions are small molecules that could enter the cell via a transport mechanism or through pores induced by AHSV NS3. Uninfected and Sf9 cells infected with wild type baculovirus therefordetermined the baseline $[^{45}Ca]^{2+}$ uptake as a result of normal and baculovirus infected cell metabolism.

At 27 h p.i. (Fig 4.7A) there was no significant difference in the ability of the cells infected with either wild type or the two recombinant baculoviruses to sequester $[^{45}Ca]^{2+}$. Approximately 10-20% of the $[^{45}Ca]^{2+}$ entered the infected cells at this time p.i. At 33 h p.i. (Fig 4.7B), cells infected with wild type baculovirus showed a slight increase in $[^{45}Ca]^{2+}$ sequestering from 8 to 20%. Cells infected with BAC-AHSV-2 82/61-NS3 sequestered more $[^{45}Ca]^{2+}$ (22 to 40%) at a slightly faster rate than that of the cells infected with wild type baculovirus. Sf9 cells infected with BAC-AHSV-2 Vac-NS3 sequestered the most $[^{45}Ca]^{2+}$ 33 h p.i. The index increased from 28 to 50% over the initial 3 min monitoring period. Influx at 36 h p.i. (Fig 4.7C) indicated that the AHSV-2 82/61 NS3 expression sequestered the most $[^{45}Ca]^{2+}$ (70 to 80%) followed by the AHSV-2 vaccine NS3 protein expression that sequestered 50 to 60%. The wild type baculovirus $[^{45}Ca]^{2+}$ sequestration index was 40%. At 48 h p.i. the most marked difference in $[^{45}Ca]^{2+}$ sequestration between the wild type and AHSV-NS3 recombinant baculovirus infected cells was noted (Fig 4.7D). The $[^{45}Ca]^{2+}$ sequestration indexes of cells expressing either the AHSV-2 82/61 or the AHSV-2 vaccine NS3 protein both bordered on 100% while the wild type baculovirus infections induced a 60% influx. These results indicate that expression of either AHSV-2 NS3 protein changes the permeability of Sf9 cell membranes but that there are no significant differences in the membrane permeabilization properties of the vaccine and virulent 82/61 strains' NS3 proteins.

Expression of NS3 in Sf9 cells was confirmed by an immunoblot using the α-AHSV-2 NS3. Results indicated that NS3 was expressed in infected Sf9 cells from 24 h p.i. onward (Fig 4.8).

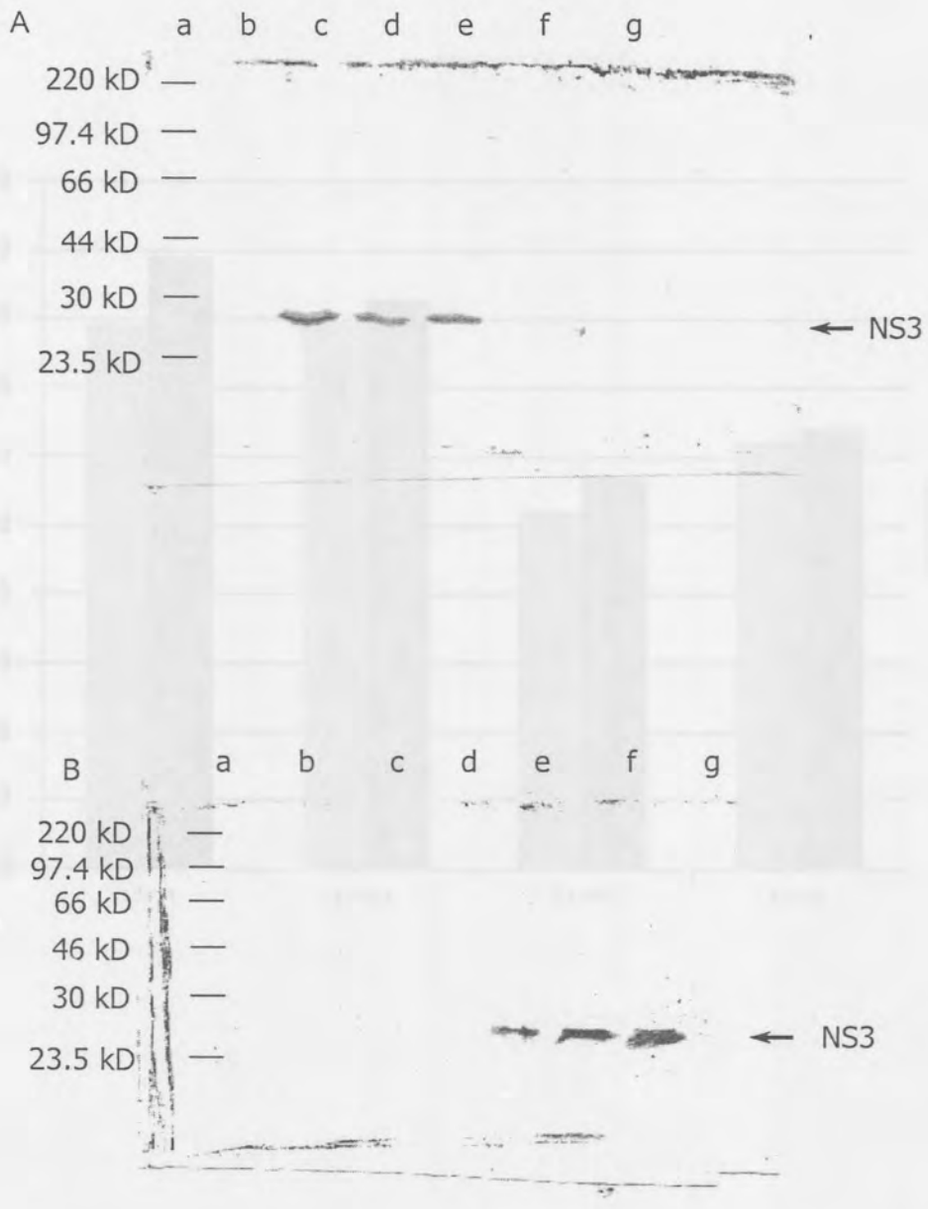


Fig 4.8 Western blot analysis to confirm AHSV-2 NS3 expression during $[^{45}Ca]^{2+}$ influx assays. (A) α-AHSV-2 NS3 serum reacted with Sf9 cell extracts infected with BAC-AHSV-2 82/61 harvested at 18 (b), 24 (c), 36 (d) and 48 (e) h p.i. Sizes of Rainbow marker are as indicated (a). Sf9 cell extracts of uninfected (f) or wild type baculovirus infected cells as controls. (B) α-AHSV-2 NS3 serum reacted with Sf9 cell extracts infected with BAC-AHSV-2 Vac-NS3 harvested at 18 (d), 24 (e), 36 (f) and 48 (g) h p.i. Sizes of Rainbow protein marker (a) and Sf9 cell extracts of uninfected (b) and wild type baculovirus infected (c) cells were included as controls.

4.3.4 Virus release and membrane permeability properties of AHSV

As previously described, AHSV NS3 protein sequences are highly variable with the greatest variation of the protein sequence observed across the NS3 clusters. It is not known if this high level of NS3 sequence variation reflected on any phenotypic and in particular on the virulence characteristics of the parental virus. To investigate this a number of AHSV strains with different NS3 genotypes were compared. Investigations at this level could also be affected by the other AHSV encoded proteins.

4.3.4.a AHSV isolates

AHSV isolates can be grouped in three phenogroups dependent on the NS3 protein for which they encode, namely α, β and γ. In order to investigate the impact of larger amount of NS3 sequence variation on virus characteristics, AHSV isolates that encoded NS3 proteins that showed a high level of variation (up to 35.6%) between them were selected;

α phenogroup - AHSV-4 39/97 (S4VIR-α)

β phenogroup – AHSV-3 m322/97 (S3VIR-β)

γ phenogroup - AHSV-2 vaccine (S2VAC-γ)

The AHSV-2 reference strain 82/61 (S2VIR-γ), from which the AHSV-2 vaccine was produced, was additionally included to compare possible differences between the NS3 proteins of these strains. All isolates were provided by the O.V.I (see Table 3.1).

The strains were plaque purified and titrated before use in experiments in which comparisons were made in terms of how efficiently virus is released from infected Vero cells and observed difference in the CPE of the infections. The virus titre in the cellular and supernatant fractions of the infections was determined at the different stages post infection and membrane permeability changes were investigated.

4.3.4.b AHSV release assays

Extracellular AHSV particles in the culture medium and those that remained associated with the cellular fraction were determined by plaque assays. Titres in either the cellular or supernatant fraction were expressed as a percentage of the combined titre. The total of the two values represented the total virus yield in the infection. The release assays were carried out in triplicate for each virus at each time interval post infection and the standard deviations were calculated.

Cells were infected at an MOI of 5 and the release of progeny AHSV particles was monitored at 24 h and 48 h p.i. (Table 4.3). The results are summarized in the form of histograms (Fig 4.9). There was no significant difference between the percentage virus released at 24 and 48 h p.i. The virulent AHSV-2 strain (S2VIR-γ) and the vaccine strain (S2VAC-γ) did not differ with respect to the percentage virus that was released but the percentage virus released (79% - 82%) was significantly higher than the release in the case of the S4VIR-α (62% - 64%) and S3VIR-β (52% - 57%).

Table 4.3 Percentage AHSV particles released into the culture medium of infected Vero cells and total virus yield as monitored at different times p.i. Standard deviations are indicated in brackets.

Virus Isolate	**Percentage virus released into the culture medium**		**Total virus yield pfu x 10^6**	
	24 h p.i	**48 h p.i**	**24 h p.i.**	**48 h p.i.**
S2VIR-γ	79 (± 4.0)	87 (± 2.6)	8.6 (± 2.4)	4.8 (± 1.7)
S2VAC-γ	80 (± 2.1)	83 (± 2.0)	7.1 (± 2.1)	29.5 (± 2.5)
S4VIR-α	62 (± 4.5)	64 (± 2.6)	2.1 (± 1.2)	1.8 (± 0.3)
S3VIR-β	52 (± 8.0)	57 (± 3.5)	2.3 (± 0.7)	9.8 (± 1.1)

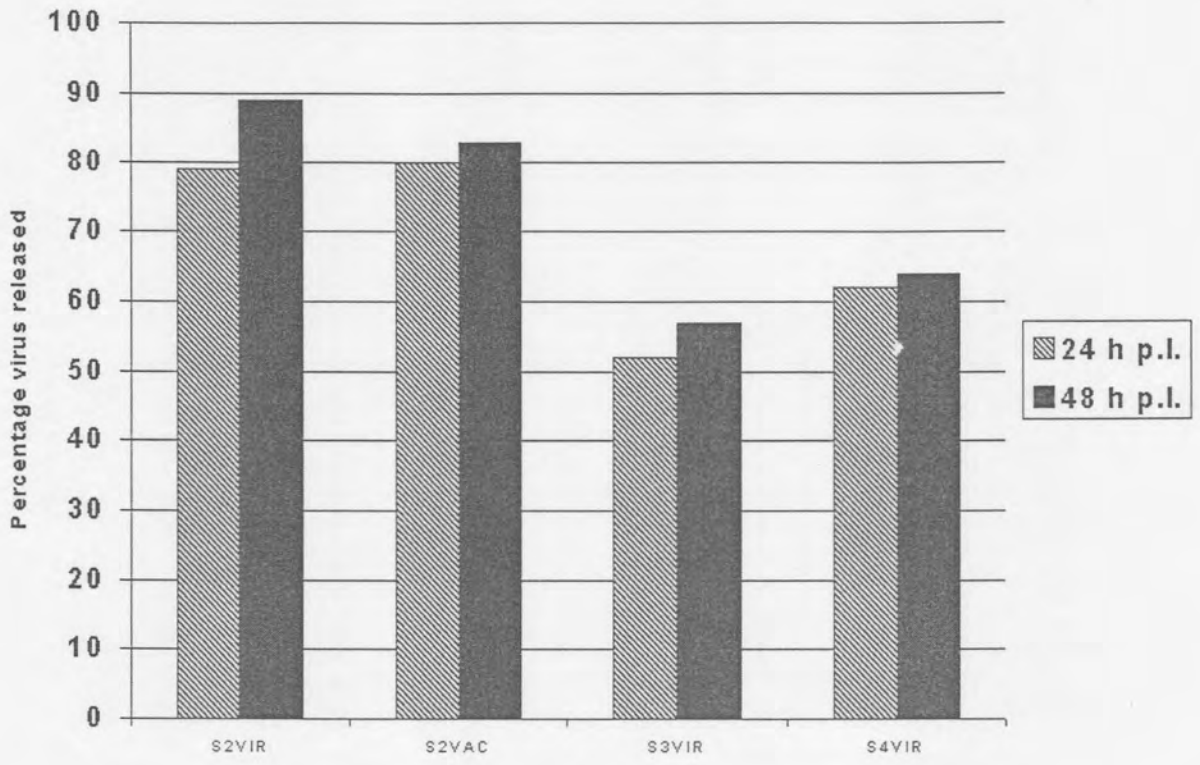


Fig 4.9 Comparison of the percentage virus released into the culture medium between the S2VIR-γ, S2VAC-γ, S3VIR-β and S4VIR-α from Vero cells infected at MOI 5.

4.3.4.c CPE on Vero cells associated with AHSV infections

In order to address the question if the percentage virus release is associated with CPE on cells, the CPE seen on the infected Vero cells was monitored and compared for the virus isolates under study (Table 4.4). CPE was scored by evaluating cell death under an inverted light microscope. Dead cells were dark, amorphic bodies that detached and drifted into the culture medium. Cell death of about 30% scored +1, 60% cell death scored +2 while 90% cell death scored +3.

At 24 h p.i. the CPE levels were found to be low. The S2VIR-γ showed a higher CPE on the Vero cells 24 h p.i. (+1) than the S2VAC-γ strain (+0.5). The highest CPE was shown on Vero cells infected with S4VIR-α (+1.5) while the S3VIR-β infections showed a CPE of +0.5. By 48 h p.i., the CPE of the two AHSV-2 strains had progressed to +3. The CPE of the S4VIR-α isolate increased to +2.5 and that of the S3VIR-β isolate to +2. At 72 h p.i. all the infections had the maximum CPE score of +3.

In general the S2VAC-γ and S2VIR-γ strains seemed to have a somewhat higher pathogenecity than the S4VIR-α and S3VIR-β strains respectively.

Table 4.4 Cytopathic effect seen on Vero cells infected with AHSV strains at an MOI of 5

Virus Isolate	Cytopathic effect		
	24 h p.i	48 h p.i	72 h p.i
S2VIR-γ	+1	+3	+3
S2VAC-γ	+0.5	+3	+3
S4VIR-α	+1.5	+2.5	+3
S3VIR-β	+0.5	+2	+3

4.3.4.c Membrane permeabilization assays

Vero cells were infected with each of the selected AHSV isolates and hygromycin B permeabilization assays (see 4.2.10) were carried out to determine whether AHSV induces a change in the permeability of the infected Vero cell plasma membranes. Hygromycin B is a protein synthesis inhibitor that can only penetrate cellular membranes after the cell membrane has been damaged or permeabilized. After hygromycin B is added to the infected cells, the cells are incubated with of [^{35}S]-methionine and percentage incorporation is measured. Reduction in protein synthesis was calculated as described and the percentage protein synthesis inhibition (Table 4.5) plotted against time (Fig 4.10).

Table 4.5 Percentage protein synthesis inhibition in mock infected Vero cells or Vero cells infected with AHSV isolates.

h p.i:	Mock	S2VAC-γ	S2VIR-γ	S4VIR-α	S3VIR- β
6	0	0	0	0	0
12	0	5	4	3	3
18	4	36	44	4	26
24	2	49	46	9	29

Mock infected cell membranes were impermeable to hygromycin B throughout the assay as protein synthesis was not inhibited and [^{35}S]-methionine incorporation remained 100% (Fig 4.10). The cells infected with the AHSV isolates however all indicated that the cellular membranes were permeable to hygromycin B as protein synthesis in each case decreased as the infection time progressed.

The AHSV-2 isolates permeabilized the membranes of the infected cells to a greater extent than that of the S4VIR-α and S3VIR-β viruses. The S2VAC-γ strains' permeabilization effect on cell membranes resulted in approximately a 36% inhibition in protein synthesis 18 h p.i. The final permeabilization effect

at 24 h p.i. resulted in 49% protein synthesis shutoff. Similar results were found for the S2VIR-γ strain. Protein synthesis was found to decrease by 44% at 18 h p.i. and remained constant at 24 h p.i. (46%).

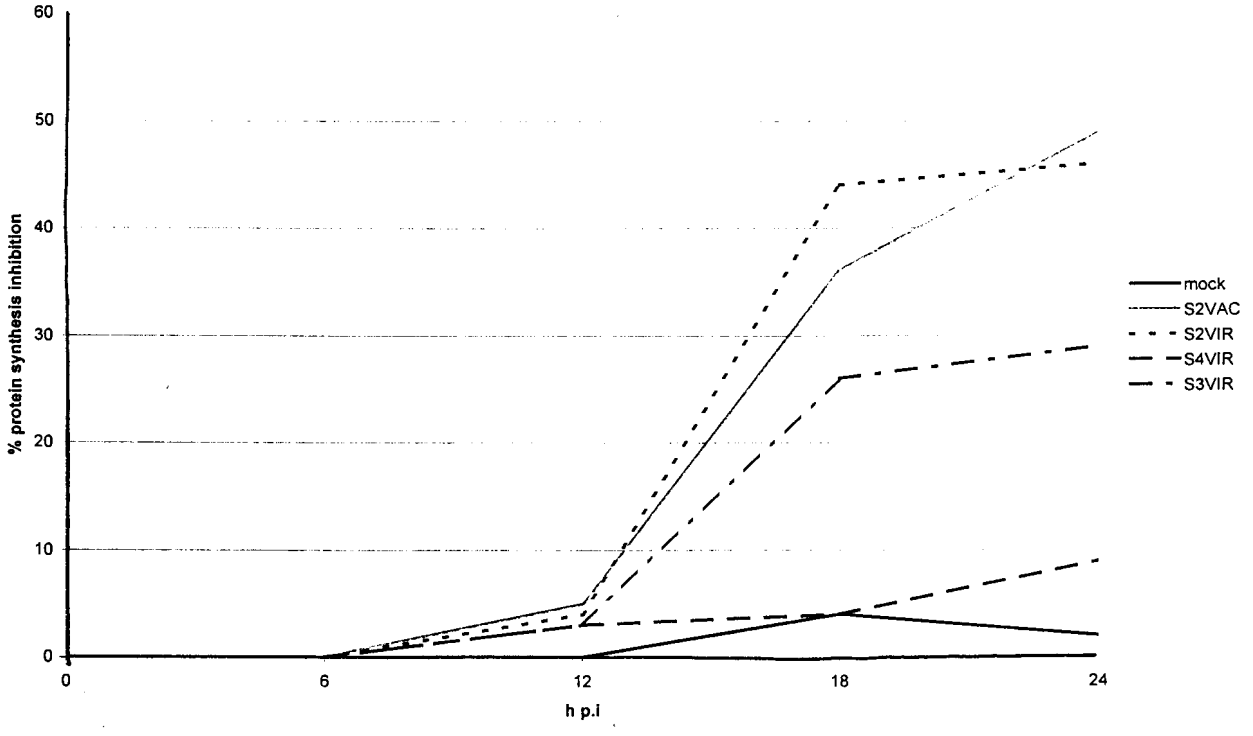


Fig 4.10 Comparison of membrane modification of the S2VIR-γ, S2VAC-γ S3VIR-β and S4VIR-α infections in Vero cells infected at MOI 3. The percentage protein synthesis inhibition was measured by a decrease in [^{35}S]-methionine incorporation in the presence of the translation inhibitor hygromycin B. Mock infected Vero cells were included as control.

The S3VIR-β had an intermediate permeabilization effect on the infected cell membranes. By 18 h p.i., the protein synthesis value had decreased by 26% and seemed to remain relatively constant being 29% at 24 h p.i. S4VIR-α infected cells' protein synthesis decreased by 9% at 24 h p.i. and appeared to have the least effect on the infected cell membranes. The decrease in protein synthesis compared to the mock infected cells was still indicative of membrane damage.

The S2VAC-γ and S2VIR-γ isolates had the greatest effect on membrane permeability followed by the S3VIR-β and S4VIR-α viruses respectively.

4.4 Discussion

The factors that determine the virulence characteristics of AHSV are likely to be highly complex and are governed by an unique combination of both host and viral factors. Virus phenotypic characteristics that are presumed to be involved in determining the virulence phenotype are the efficacy by which the virus infects the cell, replication efficacy and effective virus release from the cell. The expression of a single viral protein has also been found to contribute to virulence by altering the physiological environment in the cytoplasm. Assessment of virulence under artificial conditions is therefor likely to be oversimplified, however, parameters such as virus release and changes in cell membrane permeability may give an indication of virulence characteristics.

As discussed in Chapters 1 and 2, NS3 is one of the proteins that could play an important role in determining AHSV virulence characteristics. In this regard the observed high level of NS3 sequence variation in ASHV could become important in the virulence phenotype of the virus. To address these aspects a preliminary investigation was carried out to establish to what extent the variation level of NS3 may influence cytotoxicity.

The first question in this chapter attempted to investigate the significance of the four amino acid differences in the NS3 protein of a virulent (AHSV-2

82/61) and its attenuated avirulent (AHSV-2 vaccine) counterpart. The corresponding AHSV-2 S10 genes were cloned, expressed and used in membrane permeability assays. The detection and confirmation of the expression of AHSV-2 NS3 in infected cells requires α-AHSV-2 NS3 specific serum. Van Staden (1993) found that expression of AHSV-3 NS3 as an inducible β-gal fusion protein, when purified and injected into rabbits, successfully elicited a monospecific response against NS3 and thus the same strategy was followed to produce α-AHSV-2 NS3 serum. Serum directed against AHSV-3 NS3 failed to recognize AHSV-2 NS3. Likewise, α-AHSV-2 NS3 serum did not recognize AHSV-3 NS3 or AHSV-6 NS3. This clearly indicates the difference in immunological epitopes between the different NS3 phenogroups as a consequence of the large sequence variation.

Results obtained from the calcium influx assays indicated that AHSV NS3 expression increased the permeability of Sf9 cell membranes. Calcium cations are small molecules that may enter a cell either through active membrane transport pumps or non-specifically through small pores. Influx of calcium into cells can be a result of a number of different mechanisms. Assaying its influx does not address specific calcium transport mechanisms, but attempts to indicate changes induced in membrane permeability. Modifications in the permeability of cell membranes following virus infection and expression of viral proteins are late membrane permeability changes (Carrasco, 1994). These changes usually trigger processes leading to cell death and may include the alteration in internal pH of the infected cell, disruption of the movement of cations into or out the cell or even the permissive passage of large, non-specific molecules. Low molecular weight membrane associated proteins that increase the permeability of cell membranes and are cytotoxic to the infected cells have been termed viroporins (Carrasco, 1994). It is thought that these viroporins contribute to the overall virulence status of the specific virus. This links AHSV NS3 with viroporins and their phenotypic properties.

Calcium influx into Sf9 cells infected with baculovirus recombinants expressing NS3 was higher than that observed for Sf9 cells infected wild type baculoviruses from 33 h p.i. This time interval compares well with the cytotoxicity profiles described in the work of Smit, 1999. Smit (1999) found that Sf9 cells infected with recombinant baculoviruses expressing AHSV-3 NS3 showed enhanced trypan blue uptake from 33 h p.i. There was however no indication of a consequential difference in $[^{45}Ca]^{2+}$ influx between Sf9 cells infected with recombinant baculoviruses expressing either of the AHSV-2 NS3 proteins which could point to an enhanced membrane permeabilization effect of the AHSV-2 virulent derived NS3 protein. Cells infected with either baculovirus recombinant expressing NS3 show that approximately 100 % of the infected cell population have permeabilized membranes compared to the wild type infected cells that show an increase in membrane permeabilization of approximately 60% of the cell population. Therefor, calcium influx assays indicated that AHSV-2 NS3 permeabilizes membranes of infected cells. This is likely to contribute to its inherent cytotoxicity as described for viroporins (Carrasco, 1994). Wild type infected Sf9 cells also show a moderate level of membrane permeabilization. This may be related to the expression of baculovirus proteins that also influences the permeability of the cell membrane in the latter stage of the infection cycle or may reflect a general cytopathic effect of baculovirus on Sf9 cells. However, it is clear that expression of AHSV NS3 enhances the effect on membrane permeability.

Despite the evidence that NS3 increases cell membrane permeability, no distinct difference was found between the membrane permeabilization properties of the NS3 proteins of the AHSV-2 vaccine and virulent reference strains. These protein sequences only differed by four amino acids. This suggests that the virulence status of these parental AHSV-2 strains is not dependent on membrane permeability changes. Attenuation of the AHSV-2 82/61 is likely to be associated with viral proteins other than that of NS3. In this regard VP5 of BTV has recently been shown to increase the permeability of mammalian and *Culicoides* cell membranes (Hassan *et al.*, 2001). The four amino acid mutations observed in the vaccine strain NS3 protein are not the sole virulent determinants in these AHSV-2 isolates. This reinforces the

complexity of virulence. It is however not impossible that the attenuation process of another AHSV may be associated with changes in the S10 gene and encoding protein. This has been found for the NSP4 protein of two rotavirus strains were mutations in the VP4 viral protein binding domain of NSP4 was observed in both the corresponding attenuated viruses (Zhang *et al.*, 1998).

As the four amino acid differences in the NS3 sequences of the virulent and vaccine strains of AHSV-2 did not drastically affect the ability to increase membrane permeability it was of interest to study additional virus phenotypic properties that may affect virulence. Viral phenotypic properties that are likely to be associated with NS3 include the percentage of virus released from the cell, the CPE as well as increased membrane permeability. Membrane permeability changes are not solely related to NS3 in this assay as the system does not express NS3 exclusively but uses the entire AHSV. Results therefor obtained describe the effect of the entire virus on infected cells, which through the previous calcium influx experiments show an association with NS3.

In the case of these AHSV-2 strains only 20% of the progeny virus remained cell associated. This suggests that the different virulence status of these viruses are likely not to be attributed to efficacy of virus release from infected cells. It may be that the percentage virus released from Vero cells is not comparable to that found in the field. Additionally, these two related AHSV-2 strains did not differ in the CPE on Vero cells or in level of increased membrane permeability. The increase in membrane permeability of Vero cell did correspond with the results obtained in the calcium influx assays carried out in the Sf9 cells. The only distinctive difference between these two strains was the final virus yield in Vero cells. This occurrence is most likely to be an artifact of the attenuation process of the vaccine strain in Vero cells and has no bearing on virulence. The total of these findings suggests that NS3 is not integrally involved in the outcome of virulence in the case of the AHSV-2 vaccine and virulent strains.

The variation in AHSV NS3 is large enough to distinguish between three distinct NS3 phenogroups. The most diverse of these is the γ phenogroup while the α and β phenogroups are closer related to one another on a sequence and possibly also a functional level (see Chapter 3). Recently strains of AHSV, expressing different NS3 proteins were shown to vary in their ability to produce a fully disseminated infection in either the field vector *C. imicola* or in *C. sonorensis*, a laboratory colony (Riegler *et al.*, 2000). Riegler *et al.* (2000) postulated that the different NS3/NS3A proteins can significantly influence vector competence and therefor AHSV transmission.

The parameters used to investigate the role of the AHSV-2 NS3 proteins as a contributing role to virus virulence were expanded to investigate whether a much larger NS3 sequence variation could be linked to changes in phenotypic characteristics of the virus. Clear differences in the percentage virus released from infected cells, degree of increased membrane permeability and CPE were evident between the AHSV-2 strains and the S4VIR-α and S3VIR-β strains. These strains encode NS3 proteins that differ in sequence by 35%. Both AHSV-2 strains released 80% of the progeny virus into the culture medium while the S4VIR-α and the S3VIR-β strains released 50% - 60% of the progeny virus. The AHSV-2 strains also appeared to have a greater CPE on the cells as well as enhanced membrane permeabilization effects.

The percentage of virus released in the case of infection with S4VIR-α is slightly higher (60%) than that observed in the case of S3VIR-β (50 – 55%). The significance of such a marginal difference requires further investigation. Comparison of the virus released after infection of *Culicoides* cells, equine epithelial, lung or spleen cells may also provide a different perspective. Despite the limited difference in the virus release between the two groups, the β NS3 expressing virus had a higher membrane permeabilization affect compared to that of the α NS3 expressing virus. Comparison of the CPE of the α and β NS3 expressing viruses conversely suggested that the α NS3 expressing virus was more cytotoxic than the β NS3 expressing AHSV strain. This would therefor imply that cytotoxicity is not exclusively related to

alterations in membrane permeability, but also to other factors that could include other viral proteins.

In summary, AHSV-2 strains showed the greatest CPE, virus release and membrane damaging properties. The S4VIR-α strain had a higher CPE index and released more virus than the S3VIR-β strain, but had a smaller impact on membrane permeability. The percentage virus released is therefor not exclusively related to membrane permeabilization properties of the S4VIR-α and S3VIR-β strains. The total virus yield for these strains at 24 h p.i. was comparable. Replicative efficacy is therefore not the cause of the difference seen in membrane permeability between these two strains.

BTV is released from infected cells following cell death and subsequent lysis, by budding from infected cell membranes (Browne and Ritchie, 1970) and/or through discontinuities in the plasma membrane (Lecatsas, 1968). These events are possibly linked to expression of NS3 (Hyatt *et al.*, 1993). Previous studies on BTV virus release from MDBK-infected cells describe a BTV-8 isolate that releases virus particles as early as 4 to 5 h p.i. and has a thousand-fold excess of released compared to cell associated virus up to 15 h p.i. (Cromack *et al.*, 1971). In other cell systems a high proportion of virus remains cell associated (Howell & Verwoerd, 1971). It was also observed that in spite of the release of BTV particles by penetration of the plasma membrane, the cell membranes remained intact and that the cells were viable at time of release (Eaton *et al.*, 1980). Maximum release of BTV seemed to occur 12 – 24 h p.i. (Eaton *et al.*, 1980).

It is probable that AHSV is released from infected cells in a similar manner to that of BTV. Release also seems to occur prior to cell death and lysis as the CPE of the infected cells is still low (minimum or +1.5) with virus release ranging between 52% and 80% at 24 h p.i. A difference in virion release between certain AHSV and BTV strains may be the permeabilization of cell membranes. The AHSV-2 isolates in this study increased the permeability of the cell membranes by 24 h p.i. that correlated with 80% virus release. The

S4VIR-α strain differed in this aspect as only minimal membrane permeabilization occurred with 62% of virus being released. This may suggest that the AHSVs differ in mechanism of virus release, that may be related to different NS3 phenotypic properties.

Viral infection of host cells is a well documented form of disturbance to normal cell metabolism. Many examples can be found on the role of virus encoded membrane active toxins. HIV is an extensively characterized virus in this regard. A pathological role for HIV *env* proteins that are inserted into the plasma membrane causing its perturbation has been described. These proteins increase the rate of Ca^{2+} influx into cells, that may be responsible for depressed phospholipid and diacylglycerol synthesis, but enhanced triglyceride synthesis (Lynn *et al.*, 1988). The HIV Vpu protein is a class I integral membrane phosphoprotein that is capable of oligomerization. Two biological functions have been associated with Vpu, the first being the induction of CD4 degradation in the ER and secondly enhancement of viral release from the plasma membrane that involves two structural domains of Vpu namely the TM region and the C-terminal cytoplasmic domain (Tiganis *et al.*, 1998). Another HIV protein, Vpr, forms cation selective ion channels *in vitro* that may be important in the life cycle of HIV-1 or may cause changes in cells that contribute to AIDS-related pathogenesis (Piller *et al.*, 1996). Vpr protein added extracellularly form ion channels in the plasma lemma of neurons, causing their depolarization and eventually resulting in cell death (Piller *et al.*, 1998). The carboxyl terminus of HIV-1 and other lentiviruses TM proteins all contain a positively charged amphipathic α-helical segment. This domain, known as the lentivirus lytic peptide (LLP), exerts a toxic effect on cells through alterations in membrane integrity and permeability (Miller *et al.*, 1993). Exogenous addition of LLP to cells results in a rapid formation of distinct membrane pores leading to cell death.

Various examples can be found for other viruses illustrating the diverse mechanisms involved in membrane damage. Poliovirus 2BC (cleaved into 2B and 2C) expression leads to a number of metabolic and morphological

alterations. Expression of 2B inhibited cell growth, resulted in intracellular membrane proliferation, blocked the exocytic pathway and enhanced membrane permeability. Membrane proliferation activity is also located in 2C. The integrity of proteins 2B and 2C N-termini involved in forming amphipathic helixes are important for 2BC induced cytotoxicity (Barco & Carrasco, 1998).

The product of gene 3b of Coronavirus (single stranded positive sense RNA virus) is a nonstructural integral membrane glycoprotein of 31 kD that does not oligomerize, unlike typical viroporins, and has a postulated role in pathogenesis (O'Connor & Brian, 1999). In another example over expression of a vaccinia virus integral membrane protein, A38L, caused swelling of cells, marginalization of nuclear chromatin and vacuolization of the ER, features characteristic of cell necrosis (Sanderson *et al.*, 1996).

Hepatitis A 2C protein (integral membrane protein) inhibits growth and protein synthesis of bacteria. Deletion of the N terminal amphipathic helix of 2C abrogated this effect and its ability to associate with eukaryotic membranes. 2C also binds RNA *in vitro* and is an example of a multifunctional viral protein (Kusov *et al.*, 1998). Proteins 2B (peripheral membrane protein) and 2BC (integral membrane protein) alter membrane permeability. Another example is that of the four small hydrophobic nonstructural proteins of Japanese encephalitis virus. These proteins have various modification effects on host cell membrane permeability that contribute in part to virus-induced CPE in infected cells (Chang *et al.*, 1999).

Changes in membrane permeability could be the inciting cause for cytotoxicity or cytopathologic changes in AHSV infected cells but the overall virulence of the virus is likely to be affected by numerous other factors. These results indicate a putative role for AHSV NS3 in inducing changes in membrane permeability and therefor also cytopathology. However, it would appear unlikely that cytopathology is exclusively related to NS3 expression, but rather that NS3 is an integral component of a more complex system controlling cytopathology involving the infection process, virus tropism, replication and release.

These assays may provide a different set of results if extrapolated to another cells system. Ideally, equine or *Culicoides* cell lines would be more appropriate. It can not be ignored that the precise function of NS3 in cells remains to be established and it may be that the experimental approaches used this analysis are not the only means of addressing the question of NS3s contribution to virulence.

CHAPTER 5

CONCLUDING REMARKS

5. CONCLUDING REMARKS

The molecular and cellular basis of orbivirus virulence is an emerging field of research. The primary objective of this project was to investigate the possible contribution of AHSV NS3 towards the virulence phenotypes of AHSV.

Morphological studies of AHS lesions have indicated cellular membrane irregularities that are likely to be associated with permeability mediated oedema. Oedema manifests in various tissues and organs such as the supraorbital foci, lungs, heart, intermuscular connective tissue and the head and neck in horses infected with AHSV. Investigations have clearly indicated that AHSV NS3, the smallest nonstructural protein encoded by genome segment S10, is a cytotoxic, membrane-associated protein. Membrane association of NS3 may thus suggest that membrane irregularities observed in infected equine cells are related to the function of this specific virus encoded protein.

The first component of this study contributed to work done by Stoltz (1994) and Smit (1999) that was to investigate the structure/function relationship of specific domains of AHSV NS3. The aim of this investigation was to establish whether the hydrophobic domains of AHSV NS3 are critical for its membrane interaction. Immunofluorescent studies on Sf9 cells expressing NS3 or either of the HD mutants showed that only AHSV NS3, and not the HD mutants, was transported to, and located on the surface of infected Sf9 cells. In membrane association assays the translation of the HD mutants in the presence of canine microsomal membranes indicated that the HD mutants' ability to form stable membrane associations was abolished. From this investigation it was evident that the hydrophobic domains of AHSV NS3 are critical for membrane targeting and insertion properties of NS3 and that both these domains are required for the cytotoxic effect of NS3. Thus, the correct membrane association of NS3 is vital for its cytotoxic effect.

Membrane disruption of AHSV infected cells has been found to be mediated by NS3 that may be involved in virion release. This phenomenon is central to

the morphogenesis of other unenveloped viruses. The role of NS3 in AHSV morphogenesis is likely to be similar to that of BTV, the prototype orbivirus, where NS3 mediates the release of viral particles in the final stages of virus morphogenesis (Hyatt *et al.*, 1993). Co-expression of AHSV NS3 in cells expressing viral proteins forming CLPs and/or VLPs could establish whether NS3 also mediates the release of these particles.

In a number of cases, the cytopathic effect of an animal virus can be related to a single viral protein that increases membrane permeability of the host cell (Carrasco, 1994). These membrane-active lytic virus proteins, or viroporins, act by initially forming pores through the membrane allowing for the non-specific movement of ions and low-molecular weight compounds into or out of the cell. These events could modify the cellular ionic gradients and provide a suitable environment for virion assembly. NS3, a small integral membrane protein with two hydrophobic domains and a cluster of basic amino acids, exhibits many of the characteristics of viroporins. Another characteristic of viroporins is the formation of oligomers. Although there is no experimental evidence for multimerization of NS3, the region spanning amino acids 95 to 118 is predicted to have a high potential for forming a coiled-coil structure based on the presence of a heptad repeat motif. A similar coiled-coil domain was identified in the cytoplasmic tail of rotavirus NSP4, mediating its oligomerization (Taylor *et al.,* 1996). In general, coiled-coil regions are implicated in protein stabilization and multimerization (Cohen and Parry, 1986). Additional work is required to establish if NS3 oligomerizes or if it is monomeric in cells.

Increased cell membrane permeability as a result of NS3 expression prior to membrane disruption may contribute to its cytotoxic effect. To assess the membrane damaging potential of AHSV-2 NS3 we monitored the influx of $[^{45}Ca]^{2+}$ into Sf9 cells expressing AHSV-2 NS3. It was found that the stable expression of NS3 in Sf9 insect cells increased the permeability of the cellular membrane. This finding therefor strengthens the correlation between NS3 and viroporins. Expression of NS3 therefor increased membrane permeability thereby linking it with a classical symptom of AHS, oedema. Membrane

permeability changes mediated by NS3 may in turn cause the internal cellular environment to change thereby favouring virus replication over host metabolism. These physiological changes could be monitored to determine the basis by which NS3 alters host metabolism. Similar studies have been conducted with NSP4 of rotavirus.

It is necessary that the cytotoxic characteristics of AHSV NS3 are further investigated by expressing NS3 in cells naturally associated with AHSV such as equine and *Culicoides* cell lines. To achieve this it would be essential to express NS3 using an inducible mammalian expression system followed by a series of assays that would focus on characteristics associated with cytotoxicity. In view of the advances made in sequencing and cloning of orbivirus genes, it would be of further interest to compare the cytotoxic properties of various other orbivirus NS3 proteins. One example is that of EEV that clinically manifests in a similar manner to that of the cardiac form of AHS, but is not fatal. In this regard, cytotoxic evaluation of EEV NS3 in comparison to AHSV NS3 may provide insight into the contributing role of AHSV NS3 in virulence.

The second component of this study investigated the level of NS3 variation amongst S.A. AHSV isolates, with specific focus on identifying virulence and epidemiological markers. This study included several S.A. AHSV vaccine and reference strains as well as recent virulent field isolates. The S10 gene sequences were directly determined after RT-PCR of the S10 genes, these sequences were translated, compared and further analysed. This investigation found AHSV NS3 variation to be higher than previously reported, particularly within an AHSV serotype. Results from the NS3 sequence comparisons did not suggest that AHSV NS3 carried virulence markers. However, differences in the amino acid sequence between vaccine and virulent AHSV NS3 proteins were identified for each of the serotypes investigated here. These differences enabled us to distinguish between some current vaccine and virulent field strains of the same AHSV serotype. Additionally, it became evident that the level of NS3 variation was large enough to differentiate between virus sub-populations within a given serotype.

This is useful as an epidemiological marker and further investigations with AHSV serotype 7 isolates are underway to evaluate the specificity of NS3 in this respect. We also showed that AHSV NS3 is the second most variable virus encoded protein, the most variable protein being VP2.

The phylogenetic analysis of AHSV NS3 indicated that the sequences grouped into three distinct clusters (α, β and γ) as previously described. However, this study found more heterogenicity in the grouping of AHSV according to serotypes into these clusters that possibly indicates a greater level of S10 shifting in S.A. The inclusion of additional S.A. AHSV strains may establish the extent to which S10 shifting introduces genetic variation in AHSV.

The role of NS3 in *Culicoides* cells and why their cell membranes are not detrimentally affected by NS3 is unclear. The finding of Venter *et al.* (2000) in which certain *Culicoides* species preferentially harbour different AHSV serotypes may be linked to the variation of NS3. It would be of interest to determine the NS3 sequences of the viruses used in their study to establish whether there is any relation between the sequence variation of NS3 and insect host restriction. This may influence how AHSV spreads in the field and therefor has an impact on the epidemiology of AHSV.

As the level of S.A. AHSV NS3 variation was higher than previously found it was of interest to investigate the level of variation within S.A. BTV isolates. BTV NS3 is conserved in comparison to AHSV NS3. Despite the availability of BTV S10 and NS3 sequences, this is the first investigation to include the S10 genes and encoding NS3 proteins of a group of S.A. BTV isolates. The level of S10 and NS3 variation was only marginally higher amongst the S.A. BTV isolates than previously reported. The limited amount of S10 sequence variation between the historical BTV strains, dating back to 1900, and some recent BTV field isolates from 1999 confirmed the high level of BTV S10 and NS3 sequence conservation over a period of approximately 100 years. The phylogeny of BTV S10 indicated the existence of three BTV S10 lineages in

S.A. Inclusion of U.S.A. BTV S10 sequences in the analysis indicated that the two lineages forms part of the two clusters previously described (Pierce *et al.*, 1998). The third lineage grouped BTV strains isolated in China with recent S.A. BTV field strains. The origin of this cluster remains to be determined and would also require the sequencing of additional BTV strains isolated in Australia.

Bonneau and co-workers (1999) indicated that BTV S10 segregated BTV strains into three possible topotypes. Our investigation however found that the S.A. BTV strains clustered throughout these topotypes. Therefor, in the case of the S.A. BTV strains, one is unable to group these viruses based on geographic location. Other BTV genome segments such as L2 (Bonneau *et al.*, 1999) and S7 (Bonneau *et al.*, 2000) did not segregate BTV strains into topotypes.

The high level of AHSV NS3 variation compared to that of BTV NS3 and other AHSV proteins raises the question as to why such a large degree of variation is tolerated in AHSV NS3. The level of AHSV NS3 variation could further be brought into perspective by obtaining NS3 sequence data for numerous other *Orbivirus* members. If NS3 is a contributing factor in orbivirus virulence then the high level of AHSV NS3 variation may play a role in the manifestation of the different AHS forms.

These questions initiated comparative investigations between the NS3 proteins of the different NS3 phenogroups. We therefor investigated whether AHSV isolates that encode the different NS3 types (α, β and γ) with a high level of sequence variation between them had different phenotypic properties. Viral phenotypic properties that are possibly associated with AHSV NS3 include release of virions from infected cells, cytopathic effect, and membrane permeability changes. Results obtained from hygromycin B translation inhibition assays suggested that differences in membrane permeabilization of the selected viruses are likely to exist. We found that the AHSV-2 strains that encoded the γ NS3 protein released the highest percentage of virus from

infected cells and had the greatest membrane damaging properties. The AHSV-2 viruses differed in virulence to horses. Our studies however did not find that the virulent strain had increased virus release or membrane damaging properties compared to the vaccine strain. The only significant difference between these two strains was that the AHSV-2 vaccine virus had a higher virus yield in Vero cells. This did not effect the efficacy of virus release or membrane damaging properties of these strains and thus virus release and the increase in membrane permeability was not related to virus yield in these infections. Other viral or host factors are therefor most likely to be essential in the difference observed in the virulence of the AHSV-2 strains in this study.

The AHSV strains that encoded the α and β NS3 proteins were more similar in terms of percentage virus released. Once again the observed differences in virus yield did not seem to effect virus release as the S3VIR-β strain had a higher virus titre but released less virus the S4VIR-α strain. It was of interest that S3VIR-β had a higher membrane damaging index compared to the S4VIR-α at which time post infection the virus yield in the Vero cells were similar. Therefor, increased membrane permeability is not necessarily associated with the total virus yield in these experiments. The AHSV-2 NS3 proteins are genetically distinct from the AHSV-3 and AHSV-4 encoded NS3 proteins in this study (see Chapter 3). The AHSV-3 and AHSV-4 NS3 proteins are in turn closer related to each other. This may offer a possible explanation for the differences found in the phenotypes of the viruses in this study.

These findings may provide preliminary information that could direct future studies in elucidating the pathogenicity of AHSV. It remains to be investigated whether the different NS3 types (α, β and γ) expressed endogenously in equine cells from a recombinant expression system would induce different levels of membrane permeabilization similar to that observed in the Vero cells infected with the described AHSV strains.

It is evident that diverse mechanisms exist by which viral encoded proteins permeabilize membranes and are thereby cytotoxic. The precise function of AHSV encoded NS3 remains undefined. It is however clear that NS3 is membrane associated, involved in release of virus from infected cells and permeabilizes cell membranes with which it associates. The clinical picture of AHS involves, to a large extent, fluid imbalances seen as massive oedema and hemorrhage. These symptoms are likely to be associated with an increase in cell membrane permeability that, in our studies, correlated with the expression of NS3. Expansion of this project to fully understand the mechanism of action of AHSV NS3 in pathogenesis may ultimately lead to novel disease treatment strategies which to date are non-existent.

PARTS OF THE RESULTS IN THIS THESIS HAVE BEEN PUBLISHED:

Van Niekerk, M., Smit, C.C., Fick, W.C., Van Staden , V. and Huismans, H. (2001). Membrane association of African horsesickness virus nonstructural protein NS3 determines its cytotoxicity. *Virology* **279**, 499-508.

Van Niekerk, M., Van Staden,V., Van Dijk, A.A. and Huismans, H. (2001). Variation of African horsesickness virus nonstructural protein NS3 in southern Africa. *Journal of General Virology* **82**, 149-158.

Van Niekerk, M., Freeman, M., Guthrie, A., Howell, P.J., Paweska, J., Van Staden ,V. and Huismans, H. Genetic variation of the S10 gene and encoding nonstructural protein, NS3 of bluetongue and equine encephalosis virus in southern Africa. (Publication pending).

PARTS OF THE RESULTS IN THIS THESIS HAVE BEEN PRESENTED AT SCIENTIFIC MEETINGS:

Huismans, H., Van Niekerk, M., Meiring, T.L., Freeman, M., Lombardi, K., Van Staden, V., Van Dijk, A.A. and Guthrie, A. A comparison of nonstructural protein NS3 variation amongst isolates of African horsesickness, bluetongue and equine encephalosis viruses in southern Africa. Seventh International Symposium on Double-stranded RNA viruses, Palm Beach, Aruba, December, 2000.

Van Niekerk, M., Smit, C.C., Huismans, H. and Van Staden, V. Hydrophobic domains of African horsesickness virus nonstructural protein NS3 mediate membrane association and cytotoxicity. Seventeenth Congress of the South African Genetics Society, Pretoria, South Africa, June, 2000.

Van Niekerk, M., Van Staden ,V., Van Dijk, A.A. and Huismans, H. Large genetic variation in the NS3 gene of African horsesickness virus: Implications for virulence. XIth International Congress of Virology, Sydney, Australia, August, 1999.

Van Niekerk, M., Van Staden, V. and Huismans, H. Comparisons of the NS3 genes and gene products of a virulent and attenuated strain of AHSV-2. South African Genetics Society XVIst Congress, Bloemfontein, South Africa, July, 1998.

REFERENCES

Alexander, R.A. (1948). The 1944 epizootic of horsesickness in the Middle Aest. *Onderstepoort J Vet Res* **23**, 77-92.

Attoui, H., Stirlong, J.M., Munderloh, U.G., Billoir, F., Brookes, S.M., Burroughs, N., de Micco, P., Mertens, P.P.C. and de Lamballerie, X. (2001). Complete sequence characterization of the genome of the St Croix River virus, a new orbivirus isolated from cells of *Ixodes scapularis*. *J Gen Virol* **82**, 795-806.

Au, K. S., Chan, W. K., Burns, J. W., and Estes, M. K. (1989). Receptor activity of rotavirus nonstructural glycoprotein NS28. *J Virol* **63,** 4553-4562.

Au, K.S., Mattion,N.M., and Estes, M.K. (1993). A subviral particle binding domain on the rotavirus nonstructural glycoprotein NS28. *Virol* **194**, 665-673.

Ball, J. M., Tian, P., Zeng, C. Q., Morris, A. P., and Estes, M. K. (1996). Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein. *Science* **272,** 101-104.

Bansal, O. B., Stokes, A., Bansal, A., Bishop, D., and Roy, P. (1998). Membrane organization of bluetongue virus nonstructural glycoprotein NS3. *J Virol* **72,** 3362-3369.

Barco, A. and Carrasco, L. (1998). Identification of regions of poliovirus 2BC protein that are involved in cytotoxicity. *J Virol* **72**, 3560-3570.

Barnard, B. J. H. (1993). Circulation of African horse sickness virus in zebra (*Equus burchelli)* in the Kruger National Park, South Africa, as measured by the prevalence of type specific antibodies. *Onderstepoort J Vet Res* **60**, 111-117.

Barnard, B.J.H. (1998). Epidemiology of African horsesickness virus and the role of the zebra in South Africa. *Arch Virol [Suppl]* **14**, 13-19.

Barrage, T.G., Trevejo, R., Stone-Marschat, M. and Laegreid, W.W. (1993). Neutralizing epitopes of African horsesickness virus 4 are located on VP2. *Virol* **196**, 799-803.

Barratt-Boyes, S.M. and MacLachlan, N.J. (1994). Dynamics of viral spread in bluetongue virus infected calves. *Vet Microbiol* **40**, 361-371.

Bassel-Duby, R., Jayasuriya, A., Chatterjee, D., Soneberg, N., Maizel, J.V. and Fields, B.N. (1985). Sequence of reovirus haemagglutinin predicts a coiled-coiled structure. *Nature* **315**, 421-423.

Bentley, L., Fehrsen, J., Jordaan, F., Huismans, H. and Du Plessis D.H. (2000). Identification of antigenic regions on VP2 of African horsesickness virus serotype 3 by using phage-display epitope libraries. *J Gen Virol* **81**, 993-1000.

Billingsley, P.F. and Lehane M.J. (1996). Structure and ultrastructure of the insect midgut. *In:* Biology of the Insect midgut. pp 3-30. *Eds* P.F. Billingsley and M.J. Lehane. Chapman and Hall.

Birnboim,H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res* **7**, 1513-1523.

Bonneau, K.R., Mullens, B.A. and MacLachlan, N.J. (2001). Occurence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of

bluetongue virus upon passage between sheep, cattle and Culicoides sonorensis. *J Virol* **75**, 8298-8305.

Bonneau, K. R., Zhang, N., Zhu, J., Zhang, F., Li, Z., Zhang, K., Xiao, L., Xiang, W. & MacLachlan, N. J. (1999). Sequence comparison of the L2 and S10 genes of bluetongue viruses from the United States and the People's Republic of China. *Vir Res* **61**,153-60.

Braverman, Y. and Chizov-Ginzburg, A. (1998). Duration of repellency of various synthetic and plant-derived preparations for Culicoides immicola, the vector of African horsesickness virus. *Arch Virol [Suppl]* **14**, 165-174.

Bremer, C.W. (1976). A gel electrophoretic study of the protein and nucleic acid components of African horsesickness virus. *Onderstepoort J Vet Res* **43**, 193-20

Bressan, G.M. and Stanley, K.K. (1987). pUEX, a bacterila expression vector releated to pEX with universal host specificity. *Nucl Acid Res* **15**, 10056.

Browne, E.P., Bellamy, A.R., and Taylor, J.A. (2000). Membrane-destabilizing activity of rotavirus NSP4 is mediated by a membrane proximal amphipathic domain. *J Gen Vir* **81**, 1955-1959.

Browne, J.G. and Ritchie, A.E. (1970). Some morphological features of bluetongue virus. *Virol* **40**, 903-911.

Calisher, C.H. and Mertens, P.P.C. (1998). Taxonomy of African horsesickness viruses. *Arch Virol [Suppl]* **14**, 3-11.

Carrasco, L. (1994). Modification of membrane permeability by animal viruses. *Adv. Vir. Res.* **45**, 61-112.

Chang, K.O., Kim, Y.J., and Saif, L.J. (1999). Comparisons of nucleotide and deduced amino acid sequences of NSP4 genes of virulent and attenuated pairs of group A and C rotaviruses. *Vir Genes* **18**, 229-233.

Cho, M.W., Teterina, N., Egger, D., Bienz, K. and Ehrenfied, E. (1994). Membrane arrangement and vesicle induction by recombinant poliovirus 2C abd 2BC in human cells. *Virol* **202,** 129-145.

Ciccaglione, A.R., Marcantonio, C., Costantino, A., Equestre, M., Geraci, A. and Repicetta, M. (1998). Hepatitis C virus E1 protein induces modification of membrane permeability in *E. coli* cells. *Virol* **250**, 1-8.

Ciarlet, M., Liprandi, F., Conner, M. E. and Estes, M. K. (2000). Species specificity and interspecies relatedness of NSP4 genetic groups by comparative NSP4 sequence analyses of animal rotaviruses. *Arc Virol* **145**, 371-383.

Coetzer, J .A. and Erasmus, B. J. (1994). African horsesickness. *In* Infectious Disease of Livestock with special reference to Southern Africa. Vol 1. Chapter 44, pp. 460-475. *Eds*. J. A Coetzer, C. R. Thomson and R. C. Tustin. Eds, Oxford University Press.

Cohen, C and Parry, D. A. (1986). α-Helical coiled coils - a widespread motif in proteins. *TIBS* **11**, 245-248

Cramer, W.A., Engelman, D.M., Von Heijne, G. and Rees, D.C. (1992). Forces involved in the assembly and stabilization of membrane proteins. *The FASEB Journal* **6**, 3397-3401.

Cromack, A.S., Blue, J.L. and Gratzek, J.B. (1971). A quantative ultrastructure study of the development of bluetongue virus ib Madin-Darby bovine kidney cells. *J Gen Virol* **13**, 229-224.

DeMaula, C.D., Jutila, M.A., Wilson, D.W. and MacLachlan, N.J. (2001). Infection kinetics, prostacyclin release and cytokine modulation of the mechanism of cell death during bluetongue virus infection of cultured ovine and bovine pulmonary artery and lung microvascular endothelial cells. *J Gen Virol* **82**, 787-800.

De Sa, R. O., Zellner, M., and Grubman, M. J. (1994). Phylogenetic analysis of segment 10 from African horsesickness virus and cognate genes from other orbiviruses. *Vir Res* **33,** 157-165.

Devaney, M.A., Kendall, J. and Grubman, M.J. (1998). Characterization of nonstrucural phosphoprotein of two orbiviruses. *Vir Res* **11**, 51-64.

Doms, R. W., Lamb, R. A., Rose, J. K., and Helenius, A. (1993). Folding and assembly of viral membrane proteins. *Virol* **193,** 545-562.

Dong, Y., Zeng, C.Q., Ball, J.M., Estes, M.K., and Morris, A.P. (1997). The rotavirus enterotxin NSP4 mobilizes intacellular calcium in human intestinal cells by stimulating phospholipase C-mediated inositol 1,4,5-triphosphate production. *Proc Natl Acd Sci USA* **94**, 3960-3965.

Du Toit, R.M. (1944). The transmission of bluetongue and horsesickness by *Culicoides. Onderstepoort Vet J Res* **19**, 7-16.

Eaton, B.T., Hyatt, A.D. and Brookes, S.M. (1980). The Replication of Bluetongue virus. *Curr Top Micr Immun* **162**, 89-117.

Elliott, J., Jones, M. D., Griffin, B. E. and Krauzewicz, N. (1998). Regulation of cytoskeletal association by a basic amino acid motif in polyoma virus middle T antigen. *Oncogene* **17**, 1797-1806.

Erasmus, B.J. (1966). The attenuation of horsesickness virus: Problems and advantages associated with the use of different host systems. *Proc. 1st int. Conf. Eqine Infectious Disease*, Stresa, Italy pp. 208-213.

Erasmus, B.J. (1972). The pathogenesis of Afrcan horsesickness. *Proc. 3rd int Conf. Equine Infectious Disease*, Paris pp.1-11.

Echeverri, A.C. and Dasgupta, A. (1995). Amino terminal region of poliovirus 2C protein mediates membrane binding. *Virol* **208**, 540-553.

Fenner, F. (1979). Portraits of viruses: The poxviruses. *Intervirol* **11**, 137-157

Flint, M., Maidens, C.M., Loomis-Price, L.D., Shotton, C., Dubuisson, J., Monk, P., Higginbottom, A., Levy, J. and McKeating, J.A. (1999). Characterization of Hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. *J Virol* **73**, 6235-6244.

Flint, M. and McKeating, J.A. (1999). The C terminal region of Hepatitis C virus E1 glycoprotein confers localization within the endoplasmic reticulum. *J Gen Virol* **80**, 1943-9147.

French, T.J., Inumaru, S and Roy, P. (1989). Expression of two related nonstructural proteins of bluetongue virus (BTV) Type 10 in insect cells by a recombinant baculovirus: Production of polyclonal ascitic fluid and characterization of the gene product in BTV-infected BHK cells. *J Virol* **63**, 3270-3278.

French, T.J and. Roy, P (1990). Synthesis of bluetongue virus (BTV) corelike particles by a recombinant baculovirus expressing the two major structural core proteins. *J. Virol.* **64**, 1530-1536.

Fu, H., Leake, C.J., Mertens, P.P.C. and Mellor, P.S. (1999). The barriers of BT virus infection, dissemination and transmission in the vector *Culicoides variipennis* (Diptera: Ceratopogonidae). *Arch Virol* **144**, 1-15.

Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982). Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J Cell Biol* **93,** 97-102.

Garbutt, M., Law, L.M., Chan, H. and Hobman, T.C. (1999). Role of rubella virus glyoprotien domains in assembly of virus-like particles. *J Virol* **73**, 5324-3533.

Ghosh K. and Ghosh, H.P. (1999). The role of the membrane anchoring and cytoplasmic domains in intracellular transport and localization of viral glycoproteins. *Biochem Cell Biol* **77**, 165-178.

Ghosh, K. and Ghosh, H.P. (1999). The role of the membrane anchoring domains in intracellular transport and localization of viral glycoproteins. *Biochem Cell Biol* **77**, 165-178.

Grimes, J.M., Burroughs, J.N., Gouet, P., Diprose, J.M., Malby, R., Zientara, S., Meretens, P.P. and Stuart, D. (1998). The atomic structure of the bluetongue virus core. *Nature* **395**, 470-478.

Grubman, M.J. and Lewis, S.A. (1992). Identification and characterization of the structural and nonstructural proteins of African horsesickness virus and determination of the genome coding assignmants. *Virol* **186**, 444-451.

Guirakoo, F., Catalan, J.A. and Monath, T.P. (1995). Adaption of bluetongue virus in mosquito cell results in overexpression of NS3 proteins and release of virus particles. *Arch Virol* **140**, 967-974.

Hassan, S.H., Wirblich, C., Forzan, M. and Roy, P. (2001). Expression and functional characterization of bluetongue virus VP5 protein: role in cellular permeabilization. *J Virol* **75**, 8356-8367.

Hayama, E. and Li, J.K.-K. (1994). Mapping and characterization of antigenic epitopes and the nucleic acid-binding domains of the VP6 protein of bluetongue viruses. *J Virol* **68**, 3604-3611.

Hegde, R. S., and Lingappa, V. R. (1997). Membrane protein biogenesis: regulated complexity at the endoplasmic reticulum. *Cell* **91,** 575-582.

Hewat, E.A.,. Booth, T.F., Loudon, P.T and Roy. P. (1992). Three-dimensional reconstruction of baculovirus expressed bluetongue virus core-like particles by cryo-electron microscopy. *Virol* **189**, 10-20.

High, S., Flint, N. and Dobberstein, B. (1991). Requirements for the membrane insertion of signal-anchor type proteins. *J Cell Biol 113*, 25-34.

Hoshino, Y., Saif, L.J., Kang, S., Sereno, M.M., Chen, W. and Kapikian, A.Z. (1995). Identification of group A rotevirus genes associated with virulence of a porcine rotavirus and host range restriction of a human rotavirus in the gnotobiotic piglet model. *Virol* **209**, 274-280.

Howell, P.G. (1962). The isolation and identification of further antigenic types of African horsesickness virus. *Onderstepoort J Vet Res* **29**, 139-147.

Howell, P.G. and Verwoerd, D.W. (1971). Bluetongue virus. *In:* African swine fever, bluetongue virus. *Eds.* W.R. Hess, P.G.Howell, D.W. Verwoerd. Springer, Berlin. pp 35-74.

Howerth, E. W., Greene, C. E., and Prestwood, A. K. (1988). Experimentally induced bluetongue virus infection in white-tailed deer: coagulation, clinical pathologic, and gross pathologic changes. *Am J Vet Res* **49,** 1906-1913.

Huang, I.-J., Hayama, E., Jeong, Y.-O. and Li, J.K.-K. (1993). Conservation of the segment 4 gene sequence and of a leucine zipper motif in VP4 among five US bluetongue viruses. *Virol* **195**, 772-779.

Huismans, H. 1979. Protein synthesis in bluetongue virus-infected cells. *Virol* **92**, 385-396.

Hurley, J.V. (1978). Current views on the mechanisms of pulmonary oedema. *J Path* **125**, 59-79.

Hwang, G., Yang, Y., Chiou, J. and Li, J.K. (1992*a*). Sequence conservation among the cognate nonstructural NS3/NS3A protein genes of six bluetongue viruses. *Vir Res* **23**, 151-161.

Hwang, G., Yang, Y., Chiou, J., Yang, Y.-Y.,and Li, J.K.-K. (1992*b*). Comparative sequence analysis of the cognate structural protein VP6 genes of five US bluetongue viruses. *Vir Res* **24**, 315-323.

Hyatt, A. D., Zhao, Y. and Roy, P. (1993). Release of bluetongue virus-like particles from insect cells is mediated by BTV nonstructural protein NS3/NS3A. *Virol* **193,** 592-603.

Hyatt, A.D., Gould A.R., Coupar,B. and Eaton, B.T. (1991). Localization of the nonstructural protein NS3 in bluetongue virus-infected cells. *J Gen Virol* **72**, 2263-2267.

Jennings, D.M. and Mellor, P.S. (1987). Variation in the response of *Culicoides variipennis* to oral infection with BT virus. *Arch Virol* **95**, 177-182,

Jensen, M. J. and Wilson, W. C. (1995). A model for the membrane topology of the NS3 protein as predicted from the sequence of segment 10 of epizootic haemorrhagic disease virus serotype 1. *Arch Virol* **140**, 799-805.

Jones, D. T., Taylor, W. R. and Thornton, J. M. (1994). A model recognition approach to the prediction of all-helical membrane protein structure and topology. *Biochem* **33,** 3038-3049.

Kirkwood,C.D., Gentsch, J.R. and Glass, R.I. (1999). Sequence analysis of the NSP4 gene from human rotavirus strains isolated in the United States. *Vir Genes* **19**, 113-122.

Kirkwood, C. D. and Palombo, E. A. (1997). Genetic characterization of the rotavirus nonstructural protein, NSP4. *Virol* **236**, 258-65.

Kowalik, T.F. and Li, J.K.-K. (1991). Bluetingue virus evolution: Sequence analysis of the genomic S1 segments and major core protein VP7. *Virol* **181**, 749-755.

Kusov, Y.Y., Probst, C., Jecht, M., Jost, P.D. and Gauss-Muller, V. (1998). Membrane association and RNA binding of recombinant hepatitis A virus protein 2C. *Arch Virol* **143**, 931-944.

Laegreid, W. W., Burrage, T. G., Stone-Marschat, M. and Skowronek, A. (1992a). Electron microscopic evidence for endothelial infection by African horsesickness virus. *Vet Pathol* **29,** 554-556.

Laegreid, W. W., Skowronek, A., Stone-Marschat, M. and Burrage, T. (1993). Characterization of virulence variants of African horsesickness virus. *Virol* **195,** 836-839.

Laegreid, W.W., Stone-Marschat, M., Skowronek, A. and Burrage,T. (1992b). Infection of endothelial celss by African horsesickness viruses. *In*: Bluetongue, African horsesickness, and related orbiviruses. *Eds.* T.E. Walton and B.I. Osburn. CRC Press Boca Raton, FL. pp. 807-814.

Lecatsas, G. (1968). Electron microscopic study of the formation of bluetongue virus. *Onderstepoort J Vet Res* **35**, 139-149.

Li, J.P. and Baltimore, D. (1990). An intergenic revertant of a poliovirus 2C mutant has an uncoating defect. *J Virol* **64**, 1102-1107.

Lin, S., Niam, H.Y., Rodriguez, A.C. and Roth, M.G. (1998). Mutations in the middle of the transmembrane domain reverse the polarity of transport of the influenza virus hemagglutinin in MDCK epithelial cells. *J Cell Biol* **142**, 51-57.

Lippincott-Schwartz, J., Bonifacino, J.S., Yuan, L.C. and Klausner, R.D. (1988). Degradation from the endoplasmic reticulum: Disposing of newly synthesized proteins. *Cell* **54**, 209-220.

Lodish, H.F. (1988). Multi-spanning membrane proteins: How accurate are the models? *TIBS* **13**, 332-334.

Lopez-Rivas, A., Castrillo, J.L. and Carrasco, L. (1987). Cation content in Poliovirus-infected Hela cells. *J Gen Virol* **68**, 335-342.

Lubroth, J. (1988) African horsesickness and the epidemiomlogy in Spain. *Equine Prac* **10**, 26-33.

Lynn, W.S., Tweedale, A. and Cloyd, M.W. (1988). Human immunodefiency virus (HIV-1) cytotoxicty: Perturbation of the cell membrane and depression of phospholipid synthesis. *Virol* **163**, 43-51.

Maass D.R. and Atkinson, P.H. (1990). Rotavirus VP7, NS28 and VP4 form oligomeric structures. *J Virol* **64**,2632-2641.

MacLachlan, N.J., Rossitto, P,V., Jagels, G., Moore, P.F. and Heidner, H.W. (1990). the pathogenesis of experimental bluetongue virus infection of calves. *Vet Path* **27**, 223-229.

Maree, F.F. and Huismans, H. (1997). Characterization of tubular structures composed of nonstructural protein NS1 of African horsesickness virus expressed in insect cells. *J. Gen. Virol* **78,** 1077-1081.

Maree, S., Durbach, S., Maree F.F., Vreede, F. and Huismans, H. (1998). Expression of the major core structural proteins VP3 and VP7 of African horsesickness virus, and production of core-like particles. *Arch Virol [Suppl]* **14**, 203-209.

Martin, L. A., Meyer, A. J., O'Hara, R. S., Fu, H., Mellor, P. S., Knowles, N. J. and Mertens, P. P. (1998). Phylogenetic analysis of African horse sickness virus segment 10: sequence variation, virulence characteristics and cell exit. *Arch Virol [Suppl]* **14,** 281-293.

Mattion, N.M., Cohen, J. and Estes, M.K. (1994). Rotavirus proteins. In: *Viral infection in the Gastrointestinal Tract.* Eds. A. Kapikian. New York, Marcel Dekker. pp. 169-249.

Maurer, F.D. and McCully, R.M. (1963). African horsesickness-with emphasis on pathology. *Am J Vet Res* **24**, 235-266.

McIntosh, B. M. (1956). Complement fixation with horsesickness virus. *Onderstepoort J Vet Res***27**, 165-169.

McLaughlin, S. and Aderem, A. (1995). The myritoyl-electrostatic switch: A modulator of reversible protein-membrane interaction. *TIBS* **20**, 272-275.

Mecham, J.O. and Dean, V.C. (1988). Protein coding assignment of epizootic haemorrhagic disease virus. *J. Gen Virol.* **69**, 1255-1262.

Metcalf, H.F. and Luedke, A.J. (1980). Bluetongue and related viruses. *Bovine Pract* **15**, 188-191.

Mellor, P.S. (1990). The replication of bluetongue virus in *Culicoides* vectors. *Curr Top in Microbiol & Immunol* **162**, 143-161.

Mellor, P. S. (1993). African horsesickness: transmission and epidemiology. *Vet Res* **24**, 199-212.

Mellor, P.S., Rawlings, P., Baylis, M. and Wellby, M.P. (1998). Effect of temperature on African horsesickness virus infection in *Culicoides*. *Arch Virol [Suppl]* **14**, 155-163.

Mertens. P.P.C., Brown, F. and Sangar, D.V. (1984). Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. *J Gen Virol* **135**, 207-217.

Michelangeli, F., Ruize, M.C., delCastillo, J.R., Lubert, J.E. and Liprandi, F. (1991). Effect of rotavirus infection on intracellular calcium homeostasis in cultured cells. *Virol* **181**, 520-527.

Miller, M.A., Cloyd, M.W., Liebmann, J., Rinaldo, C.R., Islam, K.R., Wang, S.Z.S., Mietzner, T.A. and Montelaro, R.C. (1993). Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. *Virol* **196**, 89-100.

Mirazimi, A., Nilsson,M. and Svensson,L. (1998). The molecular chaperone Calnexin interacts with the NSP4 enterotoxin of rotavirus in Vivo and In Vitro. *J Virol* **72**, 8705-8709.

Newsholme, S.J. (1983). A morphological study of the lesions of African horsesickness. *Onderstepoort J Vet Res* **50**, 7-24.

Newton, K., Meyer, J. C., Bellamy, A. R. and Taylor, J. A. (1997). Rotavirus nonstructural glycoprotein NSP4 alters plasma membrane permeability in mammalian cells. *J Virol* **71,** 9458-9465.

O'Brien J.A., Taylor, J.A. and Bellamy, A.R. (2000). Probing of the structure of rotavirus NSP4: a short sequence at the extreme C terminus mediates binding to the inner capsid particle. *J Virol* **74**, 5388-5394.

O'Connor, J.B. and Brian, D.A. (1999). The major product of porcine transmissible gastroenteritis coronovirus gene 3b is an integral membrane glycoprotein of 31 kDa. *Virol* 152-161.

O'Hara, R. S., Meyer, A. J., Burroughs, J. N., Pullen, L., Martin, L. A. and Mertens, P. P. (1998). Development of a mouse model system, coding assignments and identification of the genome segments controlling virulence of African horse sickness virus serotypes 3 and 8. *Arch Virol [Suppl]* **14,** 259-279.

O'Reilly, D.R., Miller, L.K. and Luckow, V.A. (1992). Baculovirus expression vectors: A laboratory manual. W.H. Freeman and Company, New York.

Oka, T., Nakagomi, T. **and Nakagomi, O.** (2001). A lack of consistent amino aci substitutions in NSP4 between rotaviruses derived from diarrheal and asymptomatically-infected kittens. *Microbiol Immunol* **45**, 173-177.

Paabo, S., Nhat, B.M., Wold, W.S. and Peterson, P.A. (1987). A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. *Cell* **50**, 311-317.

Pelham, H.R. and Munro, S. (1993). Sorting of membrane proteins in the secretory pathway. *Cell* **75**, 603-605.

Pérez J.F., Chemello, M.E., Liprandi, F., Ruiz, M.C. and Michealangeli, F. (1998). Oncosis in MA104 cells is induced by rotavirus infection through an increase in intracellular Ca^{2+} concentration. *Virol* **252**, 17-27.

Petrie, B.L., Graham, D.Y. and Estes, M.K. (1983). Effects of tunicamycin on rotavirus morphogenesis and infectivity. *J. Virol.* **46**, 270-274.

Pierce, C. M., Balasuriya, U. B., and MacLachlan, N. J. (1998). Phylogenetic analysis of the NS3 gene of field and laboratory strains of bluetongue virus from the United States. *Virus Res* **55,** 15-27.

Piller, S.C., Edwart, G.D., Premkumar, A., Cox, G.B. and Gage, P.W. (1996). Vpr protein of human immunodefiency virus type 1 forms cation-selective channes in planar lipid bilayers. *Proc Natl Acad Sci USA* **93**, 111-115.

Piller, S.C., Jans, P., Gage, P.W. and Jans. D.J. (1998). Extracellular HIV-1 virus protein R causes a large inward current and cell death in cultured hippocampal neurons: Implications for AIDS pathology. *Proc Natl Acad Sci USA* **95**, 4595-4600.

Poruchynsky, M.S., Maass, D.R. and Atkinson, P.H. (1991). Calcium depletion blocks the maturation of rotavirus by altering the oligomerization of virus-encoded proteins in the ER. *J Cell Biol* **114**, 651-661.

Prasad B.V., Yamaguchi, S. and Roy, P. (1992). The dimensional structure of single shelled bluetongue virus. *J Virol* **66**, 2135-2142.

Riegler, L., Venter, G.J., O'Hara, R., Mellor, P.S. and Mertens, P.P. (2000). Variation in NS3 of African horsesickness can influence transmission by culicoides vector species. Seventh International Symposium on Double-stranded viruses, Palm Beach, Aruba, December, 2000.

Roner, M.R. and Joklik, W.K. (2001). Reovirus reverse genetics: Incorporation of the CAT gene into the reovirus genome. *PNAS* **98**, 8036-8041.

Roy, P., Mertens, P. P. and Casal, I. (1994). African Horse Sickness Virus Structure. *Comp. Immun. Microbiol. Infect. Dis.* **17**, 243-273.

Ruiz, M.C., Cohen, J. and Michelangeli, F. (2000). Role of Ca2+in the replication and pathogenesis of rotavirus and other viral infections. *Cell Calcium* **28**, 137-149.

Sailleau, C., Moulay, S. and Zientara, S. (1997). Nucleotide sequence comparison of the segments S10 of the nine African horsesickness virus serotypes. *Arch Virol* **142,** 965-978.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning, a laboratory manual. Cold Spring Habor. Cold Spring Laboratory Press.

Sanderson, C.M., Parkinson, J.E., Hollinshead, M. and Smith, G.I. (1996). Over expression of the vaccinia virus A38L integral membrane protein promotes Ca2+ influx into infected cells. *J Virol* **70**, 905-914.

Silverman, L. and Resh, M. D. (1992). Lysine residues form an integral component of a novel NH2-terminal membrane targeting motif for myristylated $pp60^{v\text{-}src}$. *Journal of Cellular Biology* **119**, 415-425.

Skowronek, A. J., LaFranco, L., Stone-Marschat, M. A., Burrage, T. G., Rebar, A. H. and Laegreid, W. W. (1995). Clinical pathology and hemostatic abnormalities in experimental African horsesickness. *Vet Pathol* **32,** 112-121.

Smit, C.C. (1999). Identification of critical functional domains of nonstructural protein NS3 of African horsesickness virus. MSc Thesis, Faculty of Biological and Agricultural Sciences, University of Pretoria.

Stoltz, M.A. (1994). The subcellular localization and general characteristics of nonstructural protein NS3 of African horsesickness virus. MSc Thesis, Faculty of Biological and Agricultural Sciences, University of Pretoria.

Stoltz, M. A., van der Merwe, C. F., Coetzee, J. and Huismans, H. (1996). Subcellular localization of the nonstructural protein NS3 of African horsesickness virus. *Onderstepoort J Vet Res* **63,** 57-61.

Swaggerty, C.L., Frolov, A.A., McArthur, M.J., Cox, V.W., Tong, S., Compans, R.W. and Ball, J.M. (2000). The envelope glycoprotein of simian immunodeficiency virus contains an enterotoxin domain. *Virol* **277,** 250-261.

Swanepoel, R. (1994). Rabies. *In* Infectious Disease of Livestock with special reference to Southern Africa. Vol 1. Chapter 48, pp. 493-552. *Eds*. J. A Coetzer, C. R. Thomson and R. C. Tustin. Eds, Oxford University Press.

Tan B-H., Nason, E., Staeuber, N., Jiang, W., Monastryrskaya, K. and Poy, P. (2001). RGD tripeptide of bluetongue virus VP7 protein is responsible for core attachment to *Culicoides* cells. *J Virol* **75**, 3937-3947.

Taylor, J. A., O'Brien, J. A. and Yeager, M. (1996). The cytoplasmic tail of NSP4, the endoplasmic reticulum-localized non-structural glycoprotein of rotavirus, contains distinct virus binding and coiled coil domains. *Embo J* **15,** 4469-4476.

Taylor, J.A., Meyer, J.C., Legge, M.A., O'Brien, J.A., Street, J.E., Lord, V.J., Bergmenn, C.C. and Bellamy, A.R. (1992). Transient expression and mutational analysis of the rotavirus intracellular receptor: The C-terminal methionine residue is essential for ligand binding. *J Virol* **66**, 3566-3572.

Taylor, J.A., O'Brein, J.C., Lord, V.J., Meyer, J.C. and Bellamy, A.R. (1993). The RER-localised rotavirus intracellular receptor: a truncated purified soluble form is multivalent and binds virus particles. *Virol* **194**, 807-814.

Theiler, A. (1921). African horsesickness. Department of Agriculture *Sci Bulletin* **19**, 1-32

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F and Higgins, D. G. (1997). The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nuc Acid Res* **25**, 4876-4882.

Theron, J., Uitenweerde, J.M., Huismans, H. and Nel, L.H. (1994). Comparison of the expression and phosphorylation of the non-structural protein NS2 of three different orbiviruses: evidence for the involvement of an ubiquitous cellular kinase. *J. Gen. Virol.* **75**, 3401-3411.

Tian, P., Ball, J. M., Zeng, C. Q. and Estes, M. K. (1996). The rotavirus nonstructural glycoprotein NSP4 possesses membrane destabilization activity. *J Virol* **70,** 6973-6981.

Tian, P., Estes, M.K., Hu, Y., Ball, J.M., Zeng, C.Q. and Schilling, W.P. (1995). The rotavirus nonstructurak glycoprotien NSP4 mobilizes Ca2+ from the ER. *J Virol* **69**, 5763-5772.

Tian, P., Hu, Y., Schilling, W. P., Lindsay, D. A., Eiden, J. and Estes, M. K. (1994). The nonstructural glycoprotein of rotavirus affects intracellular calcium levels. *J Virol* **68,** 251-257.

Tian P., Ottaiano, A., Reilly, P. A., Udem, S. and Zamb, T. (2000). The authentic sequence of rotavirus SA11 nonstructural protein NSP4. *Virus Research* **66**,117-122.

Tiganos, E., Friborg, J., Allain, B., Daniel, N. G., Yao, X. J. and Cohen, E. A. (1998). Structural and functional analysis of the membrane-spanning domain of the human immunodeficiency virus type 1 Vpu protein. *Virol* **251,** 96-107.

Tsai, K. and Karstad, L. (1973). The pathogenesis of epizootic hemorrhagic disease of deer: an electron microscopic study. *Am J Pathol* **70,** 379-400.

Turnbull, P.J., Cormack, S.B. and Huismans, H. (1996). Characterization of the gene encoding core protein VP6 of two African horsesickness virus serotypes. *J. Gen. Virol.* **77**, 1421-1423.

Uitenweerde, J. M., Theron, J., Stoltz, M. A. and Huismans, H. (1995). The multimeric nonstructural NS2 proteins of bluetongue virus, African horsesickness virus and Epizootic hemorrhagic disease virus differ in their single-stranded RNA-binding ability. *Virol* **209**, 624-632.

Urbano, U. and Urbano, G.F. (1994). The Reoviridae family. *Comp. Immun. Microbiol. Infect. Dis.* **17**, 151-161.

Van Dijk, A.A. and Huismans, H. (1988). In vitro transcriprion and translation of bluetongue virus mRNA. *J. Gen. Virol.* **69**, 573-581.

Van Staden, V. (1993). Characterization and expression of the gene that encodes nonstructural protein NS3 of African horsesickness virus. PhD Thesis, Faculty of Science, University of Pretoria.

Van Staden, V. and Huismans, H. (1991). A comparison of the genes which encode non-structural protein NS3 of different orbiviruses. *J Gen Virol* **72,** 1073-1079.

Van Staden, V., Smit, C. C., Stoltz, M. A., Maree, F. F. and Huismans, H. (1998). Characterization of two African horse sickness virus nonstructural proteins, NS1 and NS3. *Arch Virol [Suppl]* **14**, 251-258.

Van Staden, V., Stoltz, M. A. and Huismans, H. (1995). Expression of nonstructural protein NS3 of African horsesickness virus (AHSV): evidence for a cytotoxic effect of NS3 in insect cells, and characterization of the gene products in AHSV infected Vero cells. *Arch Virol* **140,** 289-306.

Venter, G.J., Graham, S.D. and Hamblin, C. (2000). African horse sickness epidemiology: vector competence of south african Culicoides species for virus serotypes 3, 5 and 8. *Med Vet Entomol* **12,** 245-250.

Verwoerd, D.W. (1969). Purification and characterization of bluetongue virus. *Virol* **38**, 203-212.

Verwoerd, D.W. and Erasmus, B.J. (1994). Bluetongue. *In* Infectious disease of livestock with special reference to southern Africa, Vol I. Chapter 43. pp. 443-459. *Eds.* J.A.W. Coetzer, G.R. Thomson and R.C. Tustin. Oxford University Press.

Wade-Evans, A. M. (1990). Complete sequence of genome segment 10, encoding the NS3 protein, of bluetongue virus, serotype 1 from South Africa. *Nuc Acid Res* **18**, 4920.

Walter, P. and Lingappa, V.R. (1986). Mechanisms of protein translocation across the endoplasmic reticulum membrane. *Ann Rev Cell Biol* **2**, 499-516.

Ward, R.L., Mason, B.B., Bernstein, D.I., Sander, D.S., Smith, V.E., Zandle, G.A. and Rappaport R.S. (1997). Attenuation of a human rotavirus vaccine candidate did not correlate with mutations in the NSP4 protein gene. *J Virol* **71**, 6267-6270.

Wu, V., Chen, S., Iwata, H., Compas, R. and Roy, P. (1992) Multiple glycoproteins synthesized by the smallest RNA segment (S10) of Bluetongue virus. *J Virol* **12**, 7104-7112,

Williamson, M. P. (1994). The structure and function of proline-rich regions in proteins. *Biochem J* **297,** 249-260.

Yamakawa,M., Kubo, M., and Furuuchi, S. (1999). Molecular analysis of the genome of Chuzan virus, a member of the Palyam serogroup viruses, and its phylogenetic relationships to other orbiviruses. *J Gen Virol* **80**, 937-941.

Yang, Y.-Y. and Li, J.K.-K. (1993). Glycosylation of the major outer capsid protein of bluetongue viruses. *Virol* **194**, 350-354.

Zhang, M., Zeng, C. Q., Dong, Y., Ball, J. M., Saif, L. J., Morris, A. P. and Estes, M. (1998). Mutations in rotavirus nonstructural glycoprotein NSP4 are associated with altered virus virulence. *J Virol* **72,** 3666-3672.

Zhang, M., Zeng, C. Q., Morris, A. P. and Estes, M. (2000). A functional NSP4 enterotoxin peptide secreted from rotavirus-infected cells. *J Virol* **74**, 11663-11670.

Zhou, W., Parent, L. J., Wills, J. W. and Resh, M. D. (1994). Identification of a membrane-binding domain within the amino terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. *J Virol* **68**, 2556-2569.

Zientara, S., Sailleau, C., Plateau, E., Moulay, S., Mertens, P. P. & Cruciere, C. (1998). Molecular epidemiology of African horse sickness virus based on analyses and comparisons of genome segments 7 and 10. *Arch Virol [Suppl]* **14**, 221-34.