Genetic studies of African horse sickness virus

Katarzyna Bachanek-Bankowska

Linacre College
University of Oxford

A thesis submitted for the degree of Doctor of Philosophy

Trinity 2012



The research programme was performed at the Pirbright Institute



To Tosia and Marysia

-with love

Dla Tosi i Marysi

-z miłością

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I. Abstract

African horse sickness virus (AHSV) is a ten-segmented, dsRNA virus, classified as a distinct species within the genus *Orbivirus*, family *Reoviridae.* There are nine serotypes of AHSV, any of which can cause African horse sickness (AHS), an extremely severe 'transboundary' and notifiable disease of horses, listed by OIE. AHS is currently restricted to sub-Saharan Africa, but has occasionally emerged causing major outbreaks in other geographic regions.

Complete genome nucleotide sequences were determined for nine reference-strains of AHSV (different serotypes). The selection-pressure on each genome-segment and its encoded proteins, in relation to protein function were analysed in phylogenetic comparisons. This initial AHSV sequence database also provided a basis for molecular-epidemiology studies. In particular, the role of the Seg-2 in determination of virus-serotype was used to identify viruses involved in a multi-serotype outbreak, identifying Ethiopia as an important area of AHSV circulation. Phylogenetic-relationships were also investigated between different isolates of AHSV serotypes 2, 4, 6, 8 and 9, from various locations in Africa.

Two real-time RT-PCR assays were developed for detection of the highly conserved genome segments 1 and 3, and molecular diagnosis of AHSV. Real-time RT-PCR assays were also developed, targeting Seg-2, for detection and identification of the nine AHSV serotypes. These assays are suitable for 'high throughput' characterisation of AHSV outbreak-strains, from either endemic or AHSV-free zones. The results obtained would facilitate rapid design and implementation of appropriate virus control measures.

Cross-contamination was detected in four of the original AHSV reference-strains. A new set of plaque-cloned and monotypic reference-strains was therefore identified and characterised, and is available through the Orbivirus Reference Collection (http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/AHSV-Nos.htm).
Serological relationships were analysed using antibodies against VP2 of AHSV-9 (expressed by recombinant MVA) and the nine monotypic reference-strains, showing neutralisation of both AHSV-9 and AHSV-6, in agreement with their closer phylogenetic relationship within Seg-2/VP2.

II. Acknowledgments

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VII. List of Abbreviations

A	Adenine
aa	amino acid
AGE	agarose gel electrophoresis
AGID	agar gel immuno-difusion
AHS	African horse sickness
AHSV	*African horse sickness virus*
AMV	Avian Myeloblastosis Virus
β-ME	β-2-mercaptoethanol
BHK	Baby hamster kidney
BHQ	Black Hole Quencher
bp	base pair
BrdU	5-bromo-2'-deoxyuridine
BTV	*Bluetongue virus*
C	Cytosine
cDNA	complementary DNA
CEF	chicken embryo fibroblasts
CFT	complement fixation test
CSU	Central Service Unit
LB	Luria Bertani
CO_2	Carbon dioxide
CPE	cytopathic effect
^oC	degree Celsius
DMEM	Dulbeco's modified Eagle's media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
d_N	non-synonymous
dNTP	Deoxyribonucleotide
d_S	synonymous
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
EEV	*Equine encephalosis virus*
EHDV	*Epizootic haemorrhagic disease virus*
ELISA	enzyme linked immunosorbant assays

EUBV	*Eubenangee virus*
FAM	6-Carboxyfluorescein
FBS	foetal bovine serum
FRET	fluorescence resonance energy transfer
g	gravity
G	Guanine
HiFi	High Fidelity
ICTV	International Committee on Taxonomy of Viruses
ISVP	infectious subviral particles
ITB	Inoue Transformation Buffer
l	litre
µ	micro
M	molar
m	milli
MEVP	membrane enveloped virus particles
min	minute
ML	Maximum likelihood
MOI	multiplicity of infection
MVA	modified vaccinia Ankara virus
n	nano
NCR	non-coding region
NFW	nuclease free water
ng	nanogame
NJ	Neighbour-Joining
NS	non-structural
nt	nucleotide
OBP	Onderstepoort Biological Products
OIE	Office Internationale des Epizooties
ORF	open reading frame
ORC	Orbivirus Reference Collection
PATAV	Pata virus
PCR	Polymerase chain reaction
PDAM	Park Davis Additive Medium
pfu	plaque forming unit
PHSV	Peruvian horse sickness virus

PIR	the Pirbright Institute
RdRp	RNA dependent RNA polymerase
RE	restriction enzyme
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute medium
RSA	Republic of South Africa
RT	Reverse transcription
RT-PCR	Reverse transcriptase polymerase chain reaction
rpm	resolutions per minute
SA	sialic acid
SAP	alkaline phosphatise
Seg	segment
SF	serum free
SNT	serum neutralization tests
SLAC	single-likelihood ancestor-counting
ss	Single stranded
T	Thymine
TC	transcriptase complex
TCID	Tissue culture infectious dose
TK	thymidine kinase
U	unit
UMAV	*Umatilla virus*
UV	ultraviolet
V	Volt
VIB	viral inclusion bodies
VNT	virus neutralization tests
VP	viral protein
WALV	*Wallal vir*us
WARV	*Warrego virus*
w/v	weight/volume
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

Chapter 1:
General Introduction

1.1: Introduction

African horse sickness virus (AHSV) is a double-stranded (ds) RNA virus, classified within the genus *Orbivirus*, family *Reoviridae* (Mertens *et al.*, 2005), which can infect all species of *Equidae.* The virus causes an infectious but non-contagious, insect-borne disease known as African horse sickness (AHS) that can be extremely severe in horses, but may be mild or unapparent in some other species (e.g. donkeys and zebra) (Coetzer & Guthrie, 2004). So far, nine distinct serotypes of AHSV have been identified, based on their levels of cross-reaction in serum neutralisation assays (Howell, 1962; McIntosh, 1956).

African horse sickness (AHS) is usually devastating in horses, especially in susceptible and naive populations, with mortality rates that can exceed 95% (Coetzer & Guthrie, 2004). Currently, the disease is restricted to sub-Saharan Africa, although it has occasionally spread outside this 'core region', into North Africa, the Middle East and Southern Europe (Spain and Portugal), where it persisted over a number of years (Mellor & Hamblin, 2004). The occurrence of these epizootics indicates that the virus can be transmitted in a much greater geographic region than suggested by its current distribution. Due to its economic impact and the ability of the virus to cause large 'transboundary' outbreaks of disease, AHS has been 'listed' by the Office Internationale des Epizooties (OIE) as a notifiable disease (OIE, 2011).

1.2: Classification

1.2.1: The family *Reoviridae*

African horse sickness virus represents a distinct virus species within the genus *Orbivirus*, of the family *Reoviridae*. At present the reoviruses are classified as 15 different genera, with several unassigned viruses that may represent additional genera (Attoui *et al.*, 2012). They collectively include pathogens capable of replicating in many different organisms, including phytoplankton, insects, plants, fungi, birds, reptiles, fish, shellfish and mammals (including humans) (Table 1.1) (Calisher & Mertens, 1988; Mertens *et al.*, 2005). Many of these viruses are recognized for their economic, veterinary or medical importance. Several reoviruses, including members of the genera: *Fijivirus, Oryzavirus, Phytoreovirus, Coltivirus, Orbivirus* and *Seadornavirus* are transmitted by arthropods in which they also replicate (arboviruses).

Reoviruses are usually regarded as non-enveloped, are approximately 60-80nm in diameter, with icosahedral capsids composed of up to three protein layers (Attoui *et al.*, 2012; Mellor & Mertens, 2008; Mertens *et al.*, 2005). In each case the protein capsid surrounds a central-space containing a single copy of the 9, 10, 11 or 12 linear dsRNA segments that collectively make up the viral genome (the number of genome segments depending on the virus genus) (Mertens *et al.*, 2005).

Possibly as result of a common ancestry and the fact that they face similar logistical problems in the replication of their segmented dsRNA genomes, the different reoviruses show certain marked similarities in their overall particle structure and replication strategies, (Attoui *et al.*, 2009a; Mertens & Diprose, 2004; Mertens *et al.*, 2005) (see Section 1.6).

The primary determinant of 'virus species' within the family *Reoviridae* is an ability to exchange genetic material between different virus strains of the same species during co-infection of the same cell, a process known as genome-segment 'reassortment' (Batten *et al.*, 2008b; Calisher & Mertens, 1988; Meiring *et al.*, 2009; Mertens *et al.*, 2005; O'Hara *et al.*, 1998; Potgieter *et al.*, 2009). However, direct experimental evidence of reassortment is very limited or non-existent for most of these different viruses. Therefore, in the absence of reassortment data, classification into individual virus species is based on a number of other characteristics, which reflect the degree of their relatedness, such as high levels of serological cross-reaction, or sequence similarities in conserved genome segments / proteins.

1.2.2: The genus *Orbivirus*

According to the XIXth Report of the International Committee on Taxonomy of Viruses (ICTV) the *Orbivirus* genus contains 22 different virus species and 10 tentative species which make it the largest of the 15 genera of reoviruses (Appendix I) (Attoui *et al.*, 2012; Mertens *et al.*, 2005). However, recent studies of full genome sequences of the different *Orbivirus* species have indicated need to update this classification, recognising a number of additional virus species (Belaganahalli, 2012; Belaganahalli *et al.*, 2012; Belaganahalli *et al.*, 2011; Cowled *et al.*, 2009; Vieira *et al.*, 2009).

The orbiviruses are named from the latin 'orbis' meaning ring or circle – referring to the ring shaped capsomeres on the core particle surface, as seen by negatively stained electromicroscopy. They are arboviruses that are transmitted between their respective hosts by heamatophagous arthropod vectors including insects (mosquitoes, biting midges (*Culicoides* spp.) and Phlebotomines) and/or ticks. Some species of *Orbivirus*, such as *Bluetongue virus* (BTV), *African horse sickness virus* (AHSV), *Epizootic*

haemorrhagic disease virus (EHDV), *Equine encephalosis virus* (EEV) include pathogens of high economic importance to the livestock and to equine industries.

Table 1.1: The genera of *Reoviridae* family. (from Mertens *et al*., 2005)

Genus	No. of genome segments (Genome size)	Number of species (+ unassigned or proposed species)	Hosts [Vectors]
Aquareovirus	11 (~ 23.7 kbp)	6 (+5)	Molluscs, finfish, Crustacea
Cardoreovirus	12 (~20.15 kbp)	1 (+2)	Crabs
Coltivirus	12 (~29 kbp)	2 (+1)	Mammals (including humans) [ticks]
Cypovirus	10 (~24.8 to 33.3 kbp)[a]	21 (+2)	Insects
Fijivirus	10 (~28.7 kbp)	5	Plants [delphacid planthoppers]
Dinovernavirus	9 (~23.35 kbp)	1	Mosquitoes
Idnoreovirus	10 +1[b] (~25.1 kbp)	5 (+1)	Insects
Mimoreovirus	11 (~25.56 kbp)	1	Phytoplankton
Mycoreovirus	11 or 12 (~24.4 kbp)	3	Fungi
Orbivirus	10 (~19.2 kbp)	22 (+14)	Mammals (including humans), birds [Culicoides, mosquitoes, phlebotomines, ticks]
Orthoreovirus	10 (~23.5 kbp)	5	Birds, reptiles, mammals
Oryzavirus	10 (~26.1 kbp)	2	Plants [delphacid planthoppers]
Phytoreovirus	12 (~25.1 kbp)	3 (+1)	Plants (cicidellid leafhoppers)
Rotavirus	11 (~18.5 kbp)	5 (+2)	Birds, mammals
Seadornavirus	12 (~21 kbp)	3	Mammals (including humans) [mosquitoes]
Unassigned reoviruses (potentially representing new genera)	10, 11 or 12	5	

The morphological and biochemical properties of the different orbiviruses are similar. Their genomes are composed of 10 segments of linear dsRNA, that are packaged as exactly one copy of each segment per virus particle, encoding seven structural and four non-structural proteins (Belhouchet *et al.*, 2011; Mertens *et al.*, 2005). The 'type species' of the genus *Orbivirus* (which has also been studied in the greatest depth) is *Bluetongue virus* (BTV). For this reason in some parts of this chapter, information concerning AHSV will be inferred on results from BTV studies, as a paradigm.

The different species within the genus *Orbivirus* were initially assigned on the basis of serological cross-reactions of their conserved structural and non-structural proteins (serogroups) during interactions with polyclonal sera or monoclonal antibodies, in complement fixation (CFT) and Agar gel immuno-difusion (AGID) tests, or enzyme linked immunosorbant assays (ELISA). The immuno-dominant core-particle surface-protein VP7 is the major serogroup specific antigen of the *Orbivirus* species (serogroup) showing high level cross-reactions between different isolates within an individual species (Gumm & Newman, 1982; Huismans & Erasmus, 1981; MartinezTorrecuadrada *et al.*, 1996). However, some distinct *Orbivirus* species exhibit relatively close antigenic relationships (e.g. *Bluetongue virus* (BTV), *African horse sickness virus* (AHSV), *Epizootic haemorrhagic disease virus* (EHDV), *Equine encephalosis virus* (EEV), *Eubenangee virus* (EUBV), *Palyam viru*s (PALV), *Wallal vir*us (WALV) and *Warrego virus* (WARV)). Viruses belonging to these different species/serogroups may occasionally show low-level or one-way cross-reactions in some serological assays (for example between BTV and EHDV) (Mertens *et al.*, 2005).

Comparisons of the amino acid sequence identity levels of conserved virus proteins (Attoui *et al.*, 2005; Attoui *et al.*, 2001; Belaganahalli *et al.*, 2012; Belhouchet *et al.*, 2010; Maan *et al.*, 2010b) and detection of conserved genome segments by real-time, or

conventional reverse transcription polymerase chain reactions (RT-PCR) can be used to determine the relatedness of different viruses, distinguishing between different members of the different *Orbivirus* species (serogroups) (Attoui *et al.*, 2009a; Mertens *et al.*, 2005).

1.2.3: Serotypes within individual *Orbivirus* species

The orbivirus outer capsid proteins interact with the hosts' immune systems and are therefore subjected to selective pressure (immune evasion), caused particularly by interactions with neutralizing antibodies. This has led to greater variability in the outer capsid proteins within a single *Orbivirus* species (e.g. in VP2 and VP5 of BTV and AHSV), which can be used to distinguish different orbivirus 'serotypes' in serum neutralization tests (SNT) or virus neutralization tests (VNT) (see Section 1.8.1.2 and 1.2.2) (Attoui *et al.*, 2009a; Attoui *et al.*, 2012; Mertens *et al.*, 2005).

The discrimination between different serotypes in VNT and SNT depends on the specificity of interactions between components of the outer capsid layer of the virus (VP2 and VP5 of AHSV) and the neutralising antibodies generated during infection of some mammalian hosts. The serotype of AHSV and other closely related orbiviruses is determined primarily by the larger outer capsid protein VP2 (Bremer *et al.*, 1990; Huismans & Erasmus, 1981; Mertens *et al.*, 1989b). VP2 on its own can elicit neutralizing antibodies (Calisher & Mertens, 1988; Castillo-Olivares *et al.*, 2011; Chiam *et al.*, 2009; DeMaula *et al.*, 1993; Huismans & Erasmus, 1981; Roy *et al.*, 1996; Scanlen *et al.*, 2002; StoneMarschat *et al.*, 1996) but the smaller outer coat protein, VP5, can also have some influence on serological reactions (Marshall & Roy, 1990; Martinez-Torrecuadrada *et al.*, 1999; Mertens *et al.*, 1989b).

Sequences encoding VP2 of BTV have been studied extensively and phylogenetic analyses showed that the serotype of different BTV isolates can be determined on the basis of nucleotide / amino acid identities (Hofmann *et al.*, 2008; Maan *et al.*, 2010a; Maan *et al.*, 2011b; Maan *et al.*, 2007a; Maan *et al.*, 2007b; Mertens *et al.*, 2007). Similar studies but on a smaller scale for other orbivirus species (AHSV, EHDV, EEV and Eubenangee virus (EUBV)) gave similar results (Aharonson-Raz *et al.*, 2011; Anthony *et al.*, 2009c; Belaganahalli *et al.*, 2012; Maan *et al.*, 2011a; Maan *et al.*, 2010a; Potgieter *et al.*, 2003). Seg-2 sequence data from isolates representing different serotypes of some *Orbivirus* species (BTV, AHSV, EHDV) have made it possible to developed RT-PCR tests that can distinguish between their serotypes (Hoffmann *et al.*, 2009; Maan *et al.*, 2011a; Maan *et al.*, 2010a; Mertens *et al.*, 2007; Wilson *et al.*, 2009b).

So far nine antigenically distinct serotypes of AHSV have been identified (Howell, 1962; McIntosh, 1956), although significant serological cross-relatedness was detected between AHSV serotype 1 and 2; 3 and 7; 5 and 8; 6 and 9 (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004), which may indicate closer genetic relationships (e.g. higher levels of VP2 sequence identity).

Phylogenetic analysis of nucleotide sequences for most BTV and EHDV genome segments and their deduced amino acid sequences (apart from VP7 and NS3) divides the virus isolates into groups associated with their geographic origins. The most obvious separation is into major eastern and western topotypes (Anthony *et al.*, 2009a; Gould & Pritchard, 1990; Maan *et al.*, 2010b).

1.3: The AHSV virion structure

Our understanding of AHSV structure is based on its similarities to BTV, the structure of which has been investigated extensively (Burroughs *et al.*, 1995; Grimes *et al.*, 1998; Hewat *et al.*, 1992a; Hewat *et al.*, 1992b; Nason *et al.*, 2004). BTV and AHSV virus particles are usually regarded as non-enveloped, with a three-layered icosahedral protein capsid, approximately 80 nm in diameter (Grimes *et al.*, 1998; Martin & Zweerink, 1972; Mertens *et al.*, 2005) (Figure 1.1). However, orbivirus particles can exit from infected mammalian cells, via a budding mechanism (Fu, 1995; Martin *et al.*, 1998; Noad & Roy, 2009). During this process the virus acquires a membrane envelope, generating 'membrane enveloped virus particles' (MEVP), which are likely to contain both cell derived and viral membrane proteins (NS3 and NS4) (Beaton *et al.*, 2002; Belhouchet *et al.*, 2011; Stoltz *et al.*, 1996). However, since orbivirus particles can be purified in an infectious form that does not contain this envelope (Mertens *et al.*, 1987), it has been suggested that the envelope is not required for virus transmission and may be unstable.

The AHSV core-particle (or inner capsid shell), encloses a single copy of each of the 10 dsRNA genome segments, which are packaged as a liquid crystal structure that allows the individual RNA molecules considerable freedom of movement within the capsid structure (Gouet *et al.*, 1999) and therefore permits their simultaneous independent transcription (Bremer *et al.*, 1990; Grubman & Lewis, 1992; Mertens & Diprose, 2004; Nason *et al.*, 2004; Stuart *et al.*, 1998).

The orbivirus core-particle surrounds a central 'space' that contains the dsRNA genome and a series of transcriptase complexes (TCs), each of which is composed of a single molecule of the RNA dependent RNA polymerase (RdRp) 'VP1(Pol)', a dimer of the

capping enzyme protein 'VP4(CaP)' and a hexamer of the helicase VP6(Hel) (Mertens *et al.*, 2009; Stuart *et al.*, 1998). Each of the transcriptase complexes appear to be associated with just one of the ten genome segments and is situated on the inner surface of the sub-core, just beneath a pore through the VP3 layer that is used for export of the nascent mRNA strands generated during transcription (Diprose *et al.*, 2001; Grimes *et al.*, 1998).

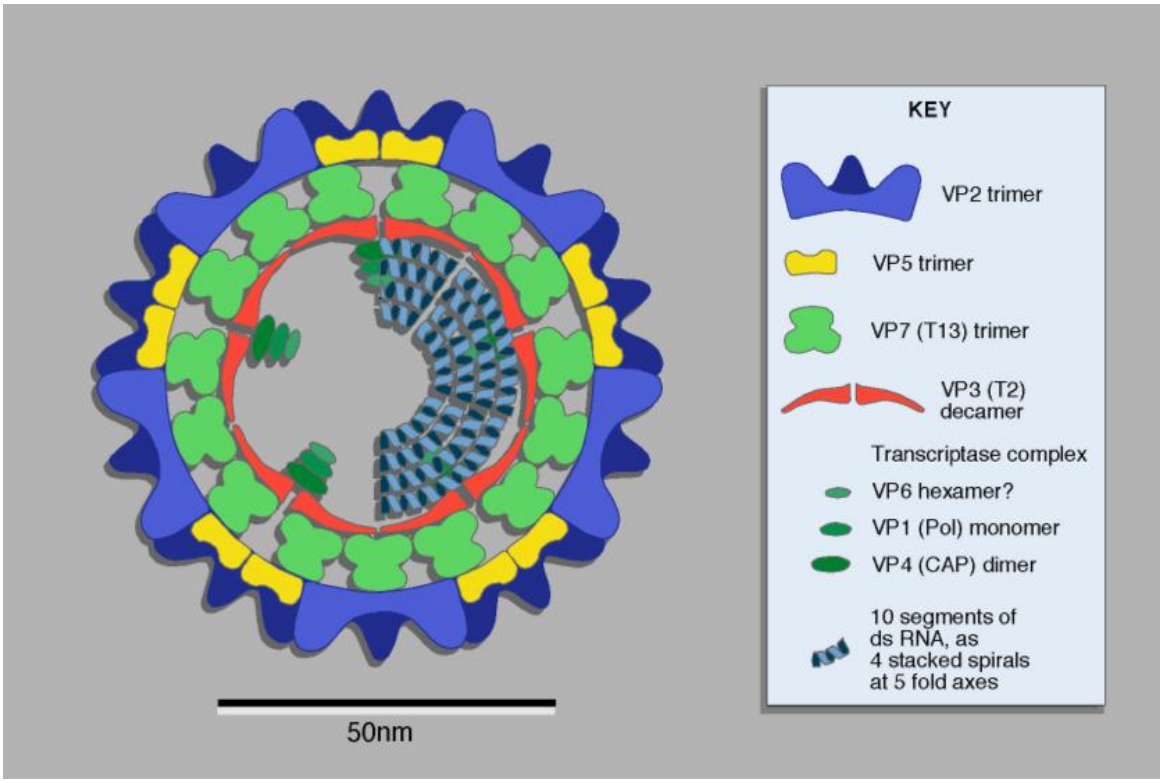


Figure 1.1: Structural organization of the AHSV particle.
The AHSV outer-capsid is composed of trimers of VP2 and VP5. The core consists of two major protein layers, the outer layer composed of VP7, is arranged on a scaffold of the inner sub-core layer, composed of the VP3. The minor structural proteins VP1, VP4 and VP6 form transcription complexes, each of which is associated with one of 10 dsRNA genome segment (from Mertens and Diprose, 2004).

The AHSV core is composed of two protein layers. The inner-most layer of the AHSV / BTV core (the sub-core shell) consists of 120 copies of VP3 (60 each of the A and B forms), which are arranged as twelve radial decamers, one centered at each of the 5-fold axes of symmetry of the icosahedrons (Figure 1.2a) (Grimes *et al.*, 1998; Mertens *et al.*, 2009; Stuart *et al.*, 1998). In the sub-core layer VP3 is organised with pseudo T=2 quasi equivalence, achieved by a conformational shift between the two otherwise chemically identical 'A' and 'B' conformers of the protein (Grimes *et al.*, 1998).

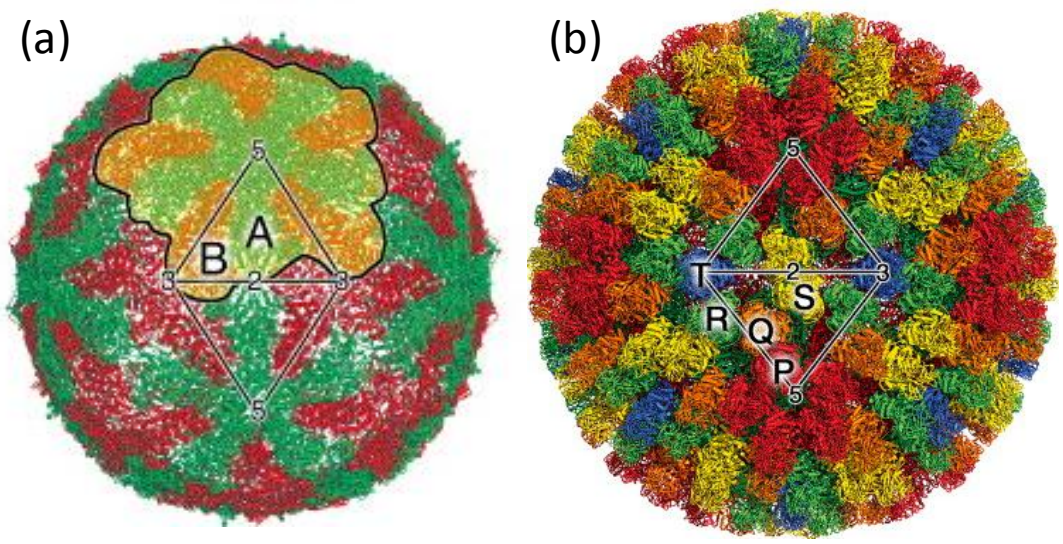


Figure 1.2: Graphical representation of VP3 and VP7 structure of BTV.
Two-fold, three-fold and five-fold symmetries of the icosahedral particle are marked. (a) Illustration of 120 molecules of VP3 of the subcore organized in T = 2 symmetry. One decamer of VP3 has been highlighted and the conformationally distinct A and B molecules are respectively indicated as green and red. (b) Illustration of the core-surface structure composed of VP7 trimers organized in T = 13 symmetry. Five different quasi equivalent trimers have been identified as P (purple), Q (orange), R (green), S (yellow) and T (blue). (Grimes *et al.*, 1998)

In contrast, the core-surface layer is composed of 260 trimers of VP7 (a total of 780 molecules), arranged with classical icosahedral T=13 symmetry, showing a considerable symmetry mismatch between the two layers of proteins that make up the core (Grimes *et al.*, 1998). Five different quasi equivalent VP7 trimers have been identified and named as P, Q, R, S and T in relation to their position to the 5-fold axis (Figure 1.2b). Inter-molecular interactions between the two layers of the core are very extensive (Grimes *et al.*, 1998; Limn & Roy, 2003) with VP3 acting as a self-assembling scaffold, supporting the surface layer of the core (VP7), which, in turn stabilizes the inner layer. In the absence of VP3, VP7 of AHSV forms flat crystal structures with in the cell cytoplasm (Burroughs *et al.*, 1994).

The outer capsid of the mature AHSV virion is assembled from two major structural proteins VP2 and VP5, arranged as a non-typical icosahedral T=13 lattice. (Figure 1.1) (Mertens & Diprose, 2004). The 360 VP5 molecules are arranged in 120 globular-shaped trimers centred on the channels formed by the hexametric rings of VP7 trimers, which may be partially shielded from the outside environment by VP2 (Noad & Roy, 2009; Zhang *et al.*, 2010). The 180 copies of VP2, arranged as 60 trimers, also interact with the VP7 molecules forming 'Y-shaped' triskellion structures, which protrude over the globular VP5 trimers (Grimes *et al.*, 1998; Hyatt & Wise, 1994; Mertens *et al.*, 2005; Nason *et al.*, 2004; Stuart *et al.*, 1998; Zhang *et al.*, 2010).

Cleavage of VP2 in the outer capsid of AHSV or BTV by serine proteases (e.g. trypsin or chymotrypsin) generates infectious subviral particles (ISVP), which (in the case of BTV) have enhanced solubility and elevated infectivity for adult *Culicoides*, or for *Culicoides* cell cultures (KC cells) (Burroughs *et al.*, 1994; Marchi *et al.*, 1995; Mertens *et al.*, 1987; Mertens *et al.*, 1996).

1.4: The AHSV genome

The *orbivirus* genome segments are numbered as segment 1 (Seg-1) to segment-10 (Seg-10) in order of decreasing molecular weight (Bremer *et al.*, 1990; Burroughs *et al.*, 1994; Gumm & Newman, 1982; Mertens *et al.*, 1989a; O'Hara, 1994; O'Hara *et al.*, 1998). The genome segment migration pattern (electropherotype) during 1% agarose gel electrophoresis (AGE) is almost identical for all serotypes of AHSV, and is similar but distinct from that of BTV or EHDV. However, the AHSV AGE-electropherotype also shows some marked similarities to that of equine encephalosis viruses (EEV) (Bremer *et al.*, 1990).

In contrast, the genome segment migration patterns of different AHSV serotypes when analysed by polyacrylamide gel electrophoresis (PAGE-electropherotype), show significant differences from each other and from those of other related orbiviruses (including BTV, EHDV and EEV) (Bremer *et al.*, 1990; O'Hara, 1994; O'Hara *et al.*, 1998). These variations reflect differences in the primary sequences of homologous genome segments, rather than differences in their molecular weights (Bremer *et al.*, 1990; Mertens *et al.*, 1989a; O'Hara, 1994). Such differences can reflect accumulation of point mutations, or insertions / deletions between isolates from different geographic locations (different topotypes) as has been described for BTV and EHDV (Anthony *et al.*, 2009a, b; Maan *et al.*, 2007a; Maan *et al.*, 2010b; Maan *et al.*, 2009; Nomikou *et al.*, 2009). However, some similarities have been detected in the PAGE electropherotype between certain isolates: between AHSV-1 and 2; AHSV-3 and 7 (Bremer *et al.*, 1990) that could reflect sequence similarities and possibly even similar genome segments.

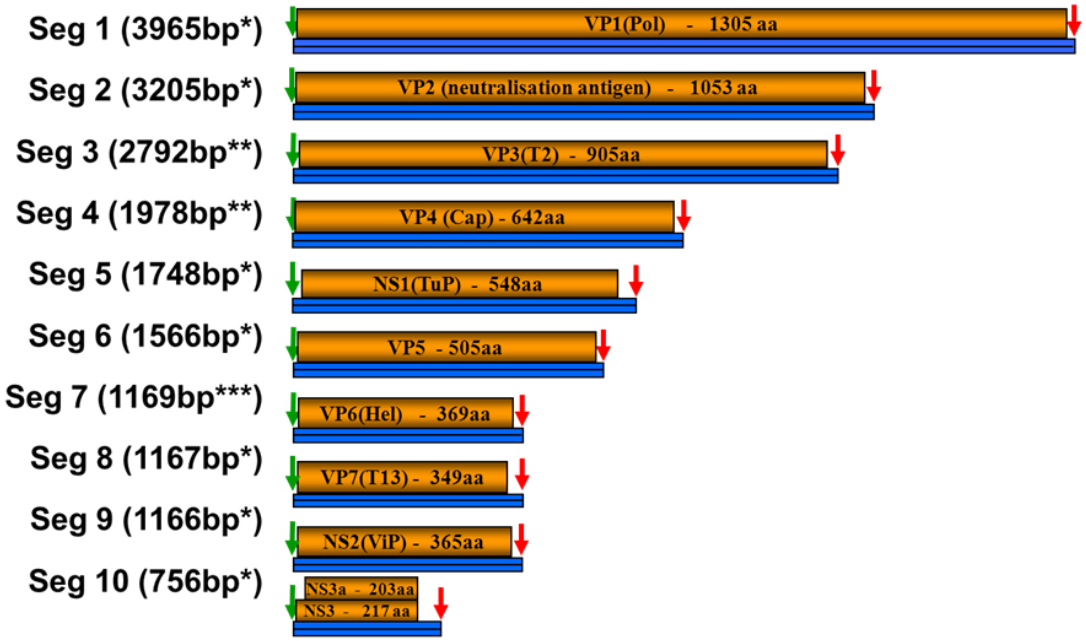


Figure 1.3: African horse sickness (AHSV) genome organization.
Individual genome segments of the virus (blue lines) encode a single protein (orange bars) with an exception of Seg-7 and Seg-10 which have two in frame ORFs respectively. The second ORF of Seg-7 (encoding NS4) has not been indicated in this illustration. Conserved terminal sequences have been indicated with green (upstream) and red (downstream).Representation of the ten genome segments of AHSV. *Data derived from AHSV-9. ** Data derived from AHSV-4. ***Data derived from AHSV-6. (Mellor & Mertens, 2008)

Comparing genome segments migration profiles in 0.8-1% AGE or 4% PAGE, to their migration patterns on PAGE gels of >6%, shows that the migration order of Seg-5 and Seg-6 was reversed (for those isolates tested) in higher percentage gels (Bremer *et al.*, 1990; O'Hara, 1994; O'Hara *et al.*, 1998). These segments are also reversed in many but not all BTV isolates (Pedley *et al.*, 1988). AHSV genome segments 7, 8 and 9 co-migrate in agarose gel electrophoresis, and all of the segments have been assigned genome segment numbers based on their molecular weights, which match their order of migration in 10% PAGE (O'Hara, 1994; O'Hara *et al.*, 1998) (Figure 1.3; Table 1.2).

The AHSV genome segments vary in size, from 3965 bp (Seg-1) to 756 bp (Seg-10), bringing the total size of the genome to approximately 19.5 kbp, depending on virus strain/serotype (Bremer *et al.*, 1990; Potgieter *et al.*, 2003). Most AHSV genome segments encode a single protein (O'Hara *et al.*, 1998; Stuart *et al.*, 1998). However, Seg-9 encodes VP6 and a recently identified non-structural protein - NS4 (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011), while Seg-10 encoded two versions of the NS3 protein (NS3 and NS3a) translated from two in frame initiation codons in the same open reading frame (ORF) (Martin *et al.*, 1998; Stoltz *et al.*, 1996).

The 5' and 3' termini of the AHSV genome segments are conserved, for all isolates of different serotypes, as: +ve strand 5'-GUU $^{A}/_{U}$ A $^{A}/_{U}$ ……AC $^{A}/_{U}$ UAC-3' (Attoui *et al.*, 2009a; Attoui *et al.*, 2012; Mertens & Rao, 2009; Potgieter *et al.*, 2003; Potgieter *et al.*, 2002) (http://www.reoviridae.org/dsRNA_virus_proteins/CPV-RNA-Termin.htm). These terminal sequences are also conserved within other *Orbivirus* species (including BTV, EEV and EHDV), although some differences have been detected between the different species (Mertens & Sangar, 1985; Potgieter *et al.*, 2002). Six conserved terminal nucleotides have also been described for BTV and for many other reoviruses (Mertens *et al.*, 2005; Mertens & Rao, 2009) (http://www.reoviridae.org/dsRNA_virus_proteins/CPV-RNA-Termin.htm). These sequences are thought to be important in recognition / recruitment / replication of viral RNAs during assembly of progeny particles (Estes & Cohen, 1989).

1.5: AHSV proteins

The number and size of the AHSV genome segment, as well as the functions of the proteins they encode, are summarized in Figure 1.1 and Table 1.2 (Mertens *et al.*, 2006).

Table 1.2: AHSV genome segments and proteins.
(Belaganahalli *et al.*, 2011; Mertens *et al.*, 2006; Ratinier *et al.*, 2011).

Genome segment	Size (bp)	Protein code	Location (number of copies per virion) function
1	3965	VP1	Core associated (10), RNA dependent RNA polymerase. Conserved.
2	3205	VP2	Outer capsid (180, trimer), serotype specific antigen, cell attachment protein, co-determines virulence, cleaved by proteases. The most variable.
3	2792	VP3	Inner layer of capsid (120, decamer) T=2 symmetry, scaffold for VP7. Highly conserved.
4	1978	VP4	Core associated (20, dimer), capping enzyme. Conserved.
5	1748	NS1	Non-structural protein (0), Forms tubular structures in cytoplasm characteristic of unknown function. Conserved.
6	1566	VP5	Outer capsid (360, trimers), co-defining serotype. Variable.
7	1169	VP6	Core associated (72 hexsamer), RNA helicase. Conserved.
		NS4	Non-structural protein (0), plays a role in virus host interactions, localises in the nucleoli of infected cells.
8	1167	VP7	Outer core surface (780, trimer) T=13 symmetry, serogroup specific, involved in cell entry, form flat hexagonal crystals made up of layers of hexameric rings of trimers, involved in high core infectivity in vector insect and cells. Conserved.
9	1166	NS2	Non-structural protein (0), forms virus inclusion body, sometimes associated with outer capsid. Conserved.
10	756	NS3	Non-structural protein (0), membrane protein, aids cell exit of virus particles by budding, may be involved in determination of virulence, cytotoxic. Highly variable.
		NS3a	Translated from a 2nd, in frame, start codon (AUG).

1.5.1: AHSV outer capsid proteins (VP2 and VP5)

The outermost surface of the AHSV virion is composed of VP2 and VP5 (as described in Section 1.3) which are involved in cell attachment and initiation of infect`ion in mammalian and insect cells (Hassan & Roy, 1999). VP2 is the primary determinant of

AHSV and BTV serotype, controlling interactions between neutralizing antibodies and the virus particle (Bentley *et al.*, 2000; Burrage *et al.*, 1993; Castillo-Olivares *et al.*, 2011; Huismans & Erasmus, 1981; Kahlon *et al.*, 1983; Maan *et al.*, 2007a; Martinez-Torrecuadrada & Casal, 1995; Mertens *et al.*, 2007; Vreede & Huismans, 1994). VP2 is also the most exposed protein of the outer capsid (Nason *et al.*, 2004; Zhang *et al.*, 2010) and, possibly due to interactions with the hosts' immune systems, the most variable (Anthony *et al.*, 2009c; Huismans *et al.*, 2004; Iwata *et al.*, 1992; Maan *et al.*, 2008; Maan *et al.*, 2007a; Maan *et al.*, 2010b; Maan *et al.*, 2009; Potgieter *et al.*, 2003; Pritchard & Gould, 1995; Vreede & Huismans, 1994). Consequently, Seg-2 (encoding VP2) and to a lesser extent Seg-6 (encoding VP5), vary in a serotype-specific manner (Anthony *et al.*, 2009c; Maan *et al.*, 2010b; Potgieter *et al.*, 2003). For these reasons, Seg-2 has been a focus of phylogenetic and molecular epidemiology studies, especially of BTV (Cêtre-Sossah *et al.*, 2011; Hofmann *et al.*, 2008; Maan *et al.*, 2011b; Maan *et al.*, 2008; Maan *et al.*, 2010b; Pritchard & Gould, 1995).

VP2 also possess haemagglutinating activity (Cowley & Gorman, 1987), plays a part in the determination of the host cell range (Coetzer & Guthrie, 2004) and is involved during virus egress (Bhattacharya *et al.*, 2007). A study with reassortant AHSV strains indicated that VP2 along with VP5 and NS3 (see Section 1.5.4) may be involved in the determination of AHSV virulence (Huismans *et al.*, 2004; O'Hara *et al.*, 1998). Recent investigations of the full genome sequence of virulent and attenuated strains of AHSV-1 have shown that segments encoding VP2, VP5 and NS3 have the highest number of differences between them (Potgieter *et al.*, 2009).

Seg-2 of all nine AHSV serotypes have been sequenced (Iwata *et al.*, 1992; Koekemoer *et al.*, 2003; Potgieter *et al.*, 2003; Sakamoto *et al.*, 1994a; Venter *et al.*, 2000b; Vreede & Huismans, 1994; Williams *et al.*, 1998) and the similarity between VP2 amino acid

sequence of the nine AHSV serotypes was calculated to stretch from 47.6% for serotypes 2 and 9, to 71.4% for serotypes 1 and 2 (Potgieter *et al.*, 2003). Phylogenetic analyses indicate that virus types which show closer serological cross reactions, also have more closely related VP2 amino acid sequences (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004; Potgieter *et al.*, 2003). Studies have been conducted to map antigenic epitopes of VP2 in AHSV serotypes 3, 4 and 9 using serological methods (Bentley *et al.*, 2000; Martinez-Torrecuadrada & Casal, 1995; Venter *et al.*, 2000b). These were later found to be located in the Seg-2 regions which are the least conserved between different serotypes of the virus (Potgieter *et al.*, 2003) (Chapter 3).

Phylogenetic analyses of the VP2 proteins of BTV and EHDV have shown that they are more closely related to each other than to those of AHSV, while AHSV is more closely related to Palyam virus (Kasba or Chuzan virus) (Potgieter *et al.*, 2003; Williams *et al.*, 1998).

The second outer capisd protein, VP5, plays an important part during the early stages of cell-entry and infection. VP5 destabilises the membrane of endosomes containing the incoming virus particles, through the activity of N-terminal α-helices, which are thought to be cytotoxic (Forzan *et al.*, 2004; Hassan *et al.*, 2001; Stassen *et al.*, 2011). Although VP2 is thought to contain the major neutralising epitopes, VP5 also contains sites recognised by neutralising antibodies (Martinez-Torrecuadrada *et al.*, 1999).

VP5 is encoded by Seg-6 (Table 1.2), containing 505 amino acids (AHSV-4) and shares 91% amino acid similarity between isolates of AHSV-4 and 6 (Williams *et al.*, 1998). Preliminary phylogenetic analyses of VP5 protein sequences and comparisons of AHSV- 4 and 6, to BTV and EHDV (using incomplete data sets) suggest that the different orbivirus serogroups represent approximately equi-distant clusters (Williams

et al., 1998). Sequence analysis of BTV and EHDV outer-capsid protein genes show "eastern" and "western" topotypic groups (Anthony *et al.*, 2009c; Maan *et al.*, 2011b; Maan *et al.*, 2008; Maan *et al.*, 2007a; Maan *et al.*, 2010b). Currently, publically available full length AHSV Seg-6 sequence data is limited and the existence of similar groupings in AHSV sequences has not yet been reported (see Section 1.10).

1.5.2: AHSV core-proteins (VP3 and VP7)

VP7(T13) is the major serogroup-specific antigen and, in its purified form, is highly immunogenic (Gumm & Newman, 1982; Huismans & Erasmus, 1981; Maree & Paweska, 2005; MartinezTorrecuadrada *et al.*, 1996). The immunogenicity of VP7 may be partly related to its high levels of expression in infected mammalian and insect cells, where it can form flat hexagonal crystals composed of 6-membered rings of VP7 trimers that reflect the organization of VP7 on the core-particle surface (Burroughs *et al.*, 1994; Chuma *et al.*, 1992).

The atomic structure of VP7 determined by x-ray crystallography shows two distinct domains within each VP7 monomer. The upper domain of VP7 interacts with the outer capsid proteins, while the lower domain is in contact with VP3 (Basak *et al.*, 1996; Basak *et al.*, 1992; Grimes *et al.*, 1998). The VP7 upper domain contains a conserved arginine-glycine-aspartic acid motif (RGD) which may be involved in cell attachment (Basak *et al.*, 1996; Tan *et al.*, 2001; Xu *et al.*, 1997). BTV core particles are infectious for *Culicoides* cells, indicating that VP7 (which forms the core surface layer, see Section 1.3) can mediate cell attachment and entry (Mertens *et al.*, 1987; Mertens *et al.*, 1996).

Exposure of intact BTV or AHSV particles to trypsin or chymotrypsin, results in cleavage of VP2, generating two or more polypeptide cleavage products that remain associated with the resulting 'infectious sub-viral particle' (ISVP) surface (Burroughs *et al.*, 1994; Mertens *et al.*, 1987). ISVP are significantly more infectious than the intact virus particle or cores, for both adult *Culicoides* and KC cell cultures (Marchi *et al.*, 1995; Mertens *et al.*, 1996), suggesting that they may represent the primary route of infection for the insect vector.

These findings were confirmed for AHSV, in an experiment where blood obtained from different animal species (ovine, bovine, equine and canine) was 'spiked' with AHSV-9 and was used to infect *C. sonorensis.* Highest infectivity was achieved with horse or dog blood, both of which are susceptible to AHSV infection, which is frequently lethal in both species. Analyses of the protein composition of the treated AHSV particles, showed that incubation with horse or dog blood had generated ISVP (Marchi *et al.*, 1995). If ISVP play a significant role in the virus life cycle, the activation and ability of host or vector proteases to cleave the virus could be an important determinant of host range (Coetzer & Guthrie, 2004; Marchi *et al.*, 1995).

VP7 of AHSV is encoded by Seg-8 and is regarded as a conserved protein, containing 354 amino acids with up to 99% identity between isolates of AHSV-4, 6 and 9 (Williams *et al.*, 1998). VP7 of AHSV, BTV and EHDV contains many hydrophobic amino-acid residues. This gives VP7 a highly insoluble nature (Basak *et al.*, 1996) and may be one of the reasons for formation of VP7 crystals within AHSV infected cells (Burroughs *et al.*, 1994). The hydrophobic character of the protein, may also be essential for its structural role and the assembly of the T=13 lattice on the relatively featureless but also hydrophobic surface of the subcore capsid shell (Grimes *et al.*, 1998). Analyses of the phylogenetic relationships between VP7 of AHSV, BTV and

EHDV confirm that BTV and EHDV are more closely related to each other than to AHSV (Williams *et al.*, 1998). Amino acid comparisons of VP7 (T13) have been used to compare different genera within the genus *Orbivirus* and establish relatedness between them (Belaganahalli *et al.*, 2012; Belaganahalli *et al.*, 2011). VP7 sequence comparisons have also been used to place novel isolates of BTV within the correct orbivirus species (Maan *et al.*, 2011b).

VP3 of AHSV is encoded by Seg-3 and is composed of 905 amino acids (for AHSV-4) (Iwata *et al.*, 1992). Analyses of the VP3 gene of AHSV, BTV and EHDV indicate that it is the most conserved of the four major capsid protein components (Iwata *et al.*, 1992; Williams *et al.*, 1998).

VP3 is the only BTV protein that can self- assemble in the absence of other viral proteins to form the sub-core shell (Kar *et al.*, 2004; Kar *et al.*, 2005). It generates a scaffold to which the outer core and outer-capsid proteins are subsequently added (Grimes *et al.*, 1998). The aa sequence of VP3 must therefore contain 'information' that determines the overall size and organisation of the core structure and subsequently of the complete virus-capsid (Mertens *et al.*, 2009). When VP3 of AHSV is co-expressed with the core-surface protein VP7 (by recombinant baculovirus), core like particles (CLP) are formed within the cell (Maree *et al.*, 1998). However, when AHSV VP7 is expressed by itself, it forms flat hexagonal crystals (Burroughs *et al.*, 1994; Chuma *et al.*, 1992), suggesting that VP3-VP7 interactions are essential for formation of the 'closed' virus-core-surface structure (Mertens *et al.*, 2009).

As described for the outer-core protein VP7(T13), amino acid comparisons of the highly conserved inner-shell protein VP3(T2) have been used to determine relatedness between different orbiviruses species (Attoui *et al.*, 2005; Attoui *et al.*, 2001;

Belaganahalli *et al.*, 2012; Belaganahalli *et al.*, 2011; Belhouchet *et al.*, 2010) and to classify novel isolates/serotypes within the appropriate species (Maan *et al.*, 2011b).

The nucleotide and amino acid sequences of Seg-3/VP3 from BTV and EHDV, like those of Seg-2/VP2 and Seg-6/VP5, show phylogenetic grouping that reflects the geographic origins of the virus isolate (Anthony *et al.*, 2009a; Maan *et al.*, 2011b; Maan *et al.*, 2008; Maan *et al.*, 2010b; Nomikou *et al.*, 2009). However, the grouping of VP7 sequences appears to be only partially related to geographic origin, with multiple eastern and western groups (Anthony *et al.*, 2009a; Maan *et al.*, 2008; Maan *et al.*, 2010b). Since VP7 can mediate the infection of insect cells, it has been suggested that grouping of VP7 sequences could be related to the insect vectors that transmit the virus (Anthony *et al.*, 2009a; Bonneau *et al.*, 2000; Maan *et al.*, 2008; Wilson *et al.*, 2000). No data is available in the literature concerning the phylogenetic grouping of VP3 or VP7 in AHSV (see section 1.10).

1.5.3: AHSV minor core proteins (VP1, VP4, VP6)

All of the minor core proteins of BTV show an “east” / “west” split in phylogenetic comparisons of nucleotide and amino acid sequences of BTV and EHDV (Anthony *et al.*, 2009a; Maan *et al.*, 2011b; Maan *et al.*, 2008; Maan *et al.*, 2010b). Similar sequence comparisons have not yet been conducted for AHSV (see Chapter 3).

VP1: is encoded by AHSV Seg-1 and, is the RNA dependant RNA polymerase (Urakawa *et al.*, 1989; Vreede & Huismans, 1998; Wehrfritz *et al.*, 2007). VP1 has both transcriptase and replicase capabilities as demonstrated through synthesis of a complementary mRNA strand from a dsRNA template, or a –ve strand from a BTV-like ssRNA template (Boyce *et al.*, 2004). VP1 is located within the viral core where the

synthesized viral RNA can be shielded from host-cell cytoplasm defence mechanisms (Mertens & Diprose, 2004).

VP1 is the largest AHSV protein, composed of 1305 amino acids (AHSV-9) and is one of the most conserved of the structural proteins, not only within individual *Orbivirus* species, but across the genus *Orbivirus*, and the family *Reoviridae* as a whole. The conserved nature of the amino acid sequence coding to the *Orbivirus* polymerase (like those of core proteins VP3(T2) and VP7(T13)) has been used, for the identification of members of the different virus species the genus and the different genera within the family (Attoui *et al.*, 2005; Attoui *et al.*, 2001; Belaganahalli *et al.*, 2012; Belaganahalli *et al.*, 2011; Belhouchet *et al.*, 2010; Maan *et al.*, 2011b).

A comparison of Seg-1 of AHSV-9 and BTV-10 showed 60.4% conservation of the nucleotide sequence and 55.6% identity in the amino acid sequences (Vreede & Huismans, 1998). The conserved nature of Seg-1 has made it a target for diagnostic group specific real-time RT-PCR assays for BTV (Shaw *et al.*, 2007) and for other orbiviruses (Palacios *et al.*, 2011).

VP4: The 5' termini of AHSV mRNA contain methylated cap structures which stabilise the viral mRNA and enable its efficient translation (Furuichi *et al.*, 1977; Furuichi & Shatkin, 1977). The methylated caps are formed by sequential enzymatic reactions mediated by the AHSV capping enzyme VP4(CaP) (Ramadevi *et al.*, 1998). VP4 has RNA triphoshatase (Ramadevi *et al.*, 1998), pyrophosphatase (Martinez-Costas *et al.*, 1998), two distinct mehyltransferase activities and a guanylyltransferase activity, which cap and methylate the viral mRNA (Le Blois *et al.*, 1992; Ramadevi *et al.*, 1998). Atomic structure studies of BTV-VP4 have revealed the protein's elongated features

with various active domains which are activated during physical passage of viral RNA through them (Sutton *et al.*, 2007).

VP4(CaP) is encoded by AHSV Seg-4 and is 642 amino acid long. A comparison of Seg-4 sequence from AHSV-4 with that of BTV-10 showed 55.4% nucleotide sequence and 48.5% amino acid identity, indicating that like VP1, it is highly conserved across the genus *Orbivirus* (Mizukoshi *et al.*, 1993). The same study describes hybridization of full-length AHSV-4 Seg-4 with the corresponding genes of AHSV serotypes 1-8. In comparisons to other AHSV serotypes, AHSV-5, 6 and 8 did not give a strong signal suggesting that although Seg-4 is conserved among all AHSV serotypes, some isolates can be more closely related to each other than others. These results indicate differences between Seg-4 sequences of individual AHSV isolates, which could potentially lead to grouping of AHSV Seg-4 sequences into discreet clades or topotypes (see Section 1.10).

VP6: In order to allow transcription by VP1(Pol), the region of the of AHSV dsRNA genome segments that is transcribed needs to be separated by the viral helicase (VP6). The same protein is also thought to mediate separation of the parental and newly synthesized RNA strands, to allowing the original strands of the genome segments to re-anneal (a fully conservative mechanism). VP6 displays ATPase, RNA binding and helicase activities (de Waal & Huismans, 2005; Roy *et al.*, 1990; Stauber *et al.*, 1997) and has been reported to bind both ss and ds RNA and DNA (de Waal & Huismans, 2005). There is evidence for regions with significant homology between VP6 of BTV and AHSV, with ATPase domains of known helicases (Hayama & Li, 1994). Like other helicases, BTV VP6 forms ring-like hexameric structures in the presence of nucleic acid, (Kar & Roy, 2003). Two forms of VP6 (VP6 and VP6a) have been reported, but

the significance of these two forms has not been investigated (O'Hara *et al.*, 1998; Wade-Evans *et al.*, 1992).

VP6 of AHSV is encoded by Seg-7, is 369 amino acids in length and is largely hydrophobic. Seg-7 has been sequenced and analysed for two strains of AHSV-3 and 6, showing that the protein is highly conserved (95% aa similarity) between the isolates examined (Turnbull *et al.*, 1996). However, comparisons of these AHSV isolates to VP6 of BTV-19 showed only 26% aa identity between virus species (less than between the other minor sub-core proteins). Recently, more sequences of Seg-7 of different serotypes of AHSV have been obtained but no such comparative studies were performed (Manole *et al.*, 2012; Potgieter *et al.*, 2009).

A bioinformatics analysis of the *Orbivirus* nucleotide and amino acid sequences (including AHSV) have indicated the presence of two independent ORFs within Seg-7 (Firth, 2008). Recently an expressed protein from the +2 ORF of Seg-7 was identified as non-structural protein 4 (NS4) (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011) (see Section 1.5.4).

1.5.4: AHSV non-structural proteins (NS1, NS2, NS3, NS4)

Four distinct non-structural (NS) proteins are synthesized during the replication of AHSV and related orbiviruses in infected insect or mammalian cells NS1(TuP), NS2(ViP) and NS3/NS3a and NS4. They are predominantly involved in the virus assembly, egress from the infected cells and control of the innate immune response (see below).

NS1: In *Orbivirus* infected cells NS1 is the most abundantly expressed protein (Van Dijk & Huismans, 1988) and assembles into characteristic elongated protein ‘tubules’,

which are morphologically and biochemically distinct from cellular filaments and tubules (Huismans & Els, 1979; Mertens *et al.*, 2005). The appearance of BTV tubules (52.3 nm in diameter and 1 µm in length) is different from the thinner and longer tubules of AHSV (23 nm in diameter and 4 µm in length) (Maree & Huismans, 1997). The AHSV 'tubule protein' NS1(TuP), is 548aa, 63.5kDa, encoded by Seg-5 (Maree & Huismans, 1997; O'Hara *et al.*, 1998). The function of tubules and of the NS1 protein has not yet been fully determined, although its early expression during BTV infection may indicate an important role in the virus replication (Hwang *et al.*, 1993; Whetter *et al.*, 1990). NS1 is located mainly in the cytoplasm of infected cells, although small amount are also present in viral inclusion bodies (VIB) (Brookes *et al.*, 1993; Eaton *et al.*, 1988). Studies have indicated that NS1 might play some role in cellular pathogenesis, possibly involving interactions with NS3/NS3a (Owens *et al.*, 2004).

A phylogenetic comparison of Seg-5 from AHSV-4 AHSV-6 and AHSV-9, showed >93% nucleotide sequence conservation and >95% amino acid identity (Maree & Huismans, 1997; Mizukoshi *et al.*, 1992). The nucleotide similarity between Seg-5 of AHSV and BTV was 49% (Maree & Huismans, 1997; Mizukoshi *et al.*, 1992).

NS2: BTV NS2 is highly expressed in infected cells and is a major component of viral the viral inclusion bodies (VIBs) that are characteristic of *Orbivirus* infected cells (Brookes *et al.*, 1993) and form as early as 4 hours after infection (Brookes *et al.*, 1993; Thomas *et al.*, 1990). VIBs are thought to be the major centres for virus replication and assembly, containing core proteins, newly synthesize viral RNAs and nascent progeny core particles (Fu, 1995; Kar *et al.*, 2007).

NS2(VIB) of AHSV-9 is encoded by Seg-9 which is 365 aa in length. NS2 amino acid sequence alignment comparisons of different orbiviruses showed that NS2 of BTV and

AHSV, AHSV and EHDV, and BTV and EHDV showed between <46% identities, between virus species (van Staden *et al.*, 1991).

NS3: Orbiviruses are released from infected host cells mainly by budding, direct membrane penetration and by cell lysis (Fu, 1995; Hyatt *et al.*, 1989; Hyatt *et al.*, 1993; Stoltz *et al.*, 1996). Genome segment 10, the smallest component of the AHSV genome (755-764 bp long – Figure 1.3 and Table 1.2), has two in frame start codons for the large ORF on the +ve strand, encoding two small non-structural proteins, NS3 (217aa) and its truncated version NS3a (Stoltz *et al.*, 1996). NS3 is a membrane glyco-protein and specifically localizes to sites of AHSV release from vero cells (Hyatt *et al.*, 1991; Stoltz *et al.*, 1996). NS3 and NS3a are expressed in insect cells and, in smaller amounts in mammalian cells (Van Dijk & Huismans, 1988). There are also reports suggesting that NS3 is involved in final stages of viral morphogenesis and release of virions (Hyatt *et al.*, 1991; Martin *et al.*, 1998; Stoltz *et al.*, 1996), and is cytotoxic to insect cells (van Niekerk *et al.*, 2001a; van Staden *et al.*, 1995). Some studies suggest a role of NS3 in viral virulence (Huismans *et al.*, 2004; Meiring *et al.*, 2009; O'Hara *et al.*, 1998) and cell membrane permeability (Han & Harty, 2004; Meiring *et al.*, 2009; van Niekerk *et al.*, 2001a).

NS3 is the second most variable AHSV protein after VP2, with sequences that can vary by up to 37% (van Niekerk *et al.*, 2001b). The significance of this high level of variation is not yet known, although as a non-structural protein, NS3 is unlikely to have any direct influence on the serological properties of the virion. Seg-10 of BTV and EEV are less variable, with 90-93% and 83% amino acid sequence conservation respectively (Anthony *et al.*, 2009b; Maan *et al.*, 2008; Maan *et al.*, 2010b; van Niekerk *et al.*, 2003). Phylogenetic comparisons of AHSV Seg-10 sequences identified three distinctive clusters α, β and γ (Martin *et al.*, 1998; Quan *et al.*, 2008; Sailleau *et al.*,

1997b; van Niekerk *et al.*, 2001b). Recently, extensive phylogenetic studies of AHSV Seg-10, detected certain serotypes within a single cluster (6, 8 and 9 in α; 3 and 7 in β; 2 in γ), while other serotypes (1, 4 and 5) grouped in both clusters α and γ (Quan *et al.*, 2008). Although this could reflect the analysis of only a limited group of AHSV samples, it also suggests that certain versions of the NS3/NS3a proteins may be 'restricted' to specific AHSV serotypes, the identity of which is controlled by variations in outer capsid proteins VP2 and VP5. It is therefore possible that this 'restriction' indicates a significant requirement for functional interactions between NS3/NS3a and the outer capsid components (Celma & Roy, 2009).

Phylogenetic analysis of the nt and aa sequences of the different non-structural proteins of BTV and EHDV have shown both eastern and western geographic groups (topotypes) in the sequences encoding NS1 and NS2 (Anthony *et al.*, 2009b; Maan *et al.*, 2008; Maan *et al.*, 2010b). However, like VP7, NS3 shows only a partial correlation to the geographic origin of the isolates (Anthony *et al.*, 2009b; Balasuriya *et al.*, 2008; Cêtre-Sossah *et al.*, 2011; Gould & Pritchard, 1990; Maan *et al.*, 2011b; Maan *et al.*, 2008; Maan *et al.*, 2010b; MacLachlan *et al.*, 2007; Nikolakaki *et al.*, 2005). It has been suggested that variations in the sequences of genome encoding VP7 and NS3 could be related to transmission by different insect vector species or by different (divergent) vector populations (Bonneau *et al.*, 2000; Maan *et al.*, 2008; Wilson *et al.*, 2000). Phylogenetic grouping of AHSV NS3, as explained above, also show groupings (which could reflect different vector species) but the larger scale relationships between sequences of NS1 and NS2 of AHSV that might reflect topotypes, have not yet been investigated (see Section 1.10 and Chapter 3) .

NS4: Recently, a novel non-structural protein 4 (NS4) has been described in BTV and Great Island Virus, encoded by the same genome segment as VP6, from a downstream

and overlapping +2 ORF (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011). Bioinformatic analyses have also indicated the presence of a functional NS4 ORF in most other orbiviruses, including AHSV (Belhouchet *et al.*, 2011; Firth, 2008). NS4 is expressed early in the replication at 4 hours post-infection and, at first, localises as aggregates in the cytoplasm (Belhouchet *et al.*, 2011). At later stages of infection (at 72 hours pi) NS4 was also located in the nucleus (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011). This novel protein was also found to associate with cell membranes which suggests a role in virus cell-exit (Belhouchet *et al.*, 2011). However NS4 was found dispensable for replication in both insect and mammalian cell cultures (Ratinier *et al.*, 2011). An involvement of NS4 in virus-host interactions has been suggested as BTV-8 NS4 was found to counteract antiviral responses of the host (Ratinier *et al.*, 2011). It also prevents DNA degradation by DNase, but does not prevent RNase III cleavage of dsRNA (Belhouchet *et al.*, 2011).

1.6: AHSV replication and morphogenesis

The replication and morphogenesis of orbiviruses takes place in both vertebrate host and arthropod vectors, implying a certain level of flexibility in these processes. The overall mechanism is represented in Figure 1.4.

Bluetongue virus infection of mammalian cell begins when the virus adsorbs to the cell surface in a process mediated by the viral outer capsid protein VP2 (Hassan & Roy, 1999). However, in insect cells the VP2 protein is dispensable for infection and the core particle can itself be infectious, which strongly suggests that orbiviruses use more than one entry mechanisms (Mertens *et al.*, 1987; Mertens *et al.*, 1996).

Core infectivity must involve interaction / attachment to the cell surface via the core-surface protein VP7. RGD motifs are recognised as integrin binding domains and have

been implicated in viral cell attachment and entry (Hynes, 1992). This family of trans-membrane α/β heterodimeric glycoproteins which modulate adhesion of molecules is also used for cell attachment and entry by other viruses including foot and mouth disease virus (Jackson *et al.*, 2000). In BTV and AHSV, an RGD-motif is exposed on the outer surface of VP7 (Basak *et al.*, 1996; Grimes *et al.*, 1998)(see Section 1.3) and that could potentially mediate BTV core particle attachment (Tan *et al.*, 2001). However BTV cores also bind to glycosaminoglycans, which are widely distributed and are present on the cell surface, but not integrins (Hutchinson, 1999).

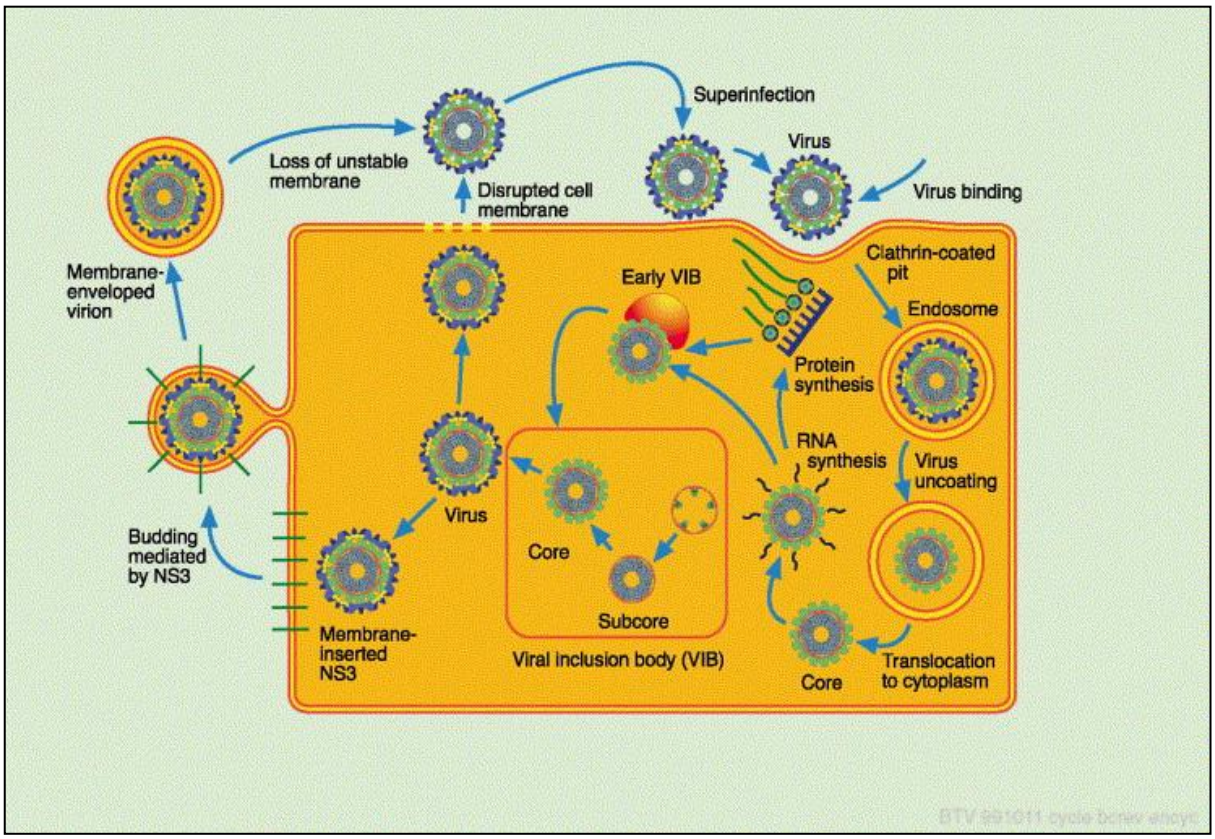


Figure 1.4: Schematic representation of the AHSV replication cycle.
(Mertens and Diprose, 2004).

Recently, sialic acid (SA) binding sites have been identified on the surface of BTV VP2 (Zhang *et al.*, 2010). The interactions of the sialic acid binding sites with the host cell surface might facilitate host cell infection through stabilisation of the virion-cell connections. In addition, previous studies have shown that BTV VP2 agglutinate erythrocytes of ruminants, equine and dogs (Eaton & Crameri, 1989; Hassan & Roy, 1999). BTV adsorption to the surface of erythrocytes could be mediated by SA binding (Zhang *et al.*, 2010). However, haemagglutinating activity is lost after proteolytic cleavage of VP2, during the formation of infectious sub-viral particles, suggesting that ISVP may bind to different receptors on the cell surface (Mertens *et al.*, 1987).

Orbivirus particles that have left the infected cell by budding have a membrane envelope (Fu, 1995; Martin *et al.*, 1998; Noad & Roy, 2009). It is not clear if these MEVP are also infectious in their own right, but it is possible that the membrane and the viral and/or cellular proteins that it contains could influence virus transmission and cell entry, which could therefore be affected by the nature of the host cell in which the virus last replicated (e.g. insect or mammalian).

The cell binding receptors for BTV or AHSV in either insect or mammalian cells are not yet defined. The BTV virus particle can enter the host cell via receptor-mediated endocytosis, a process that requires formation of clathrin-coated vesicles, which fuses with endosomes. BTV was observed in early endosomes (Eaton *et al.*, 1988). The low pH environment of endosomes triggers the removal of VP2 and the fusion of VP5 with the membrane of the vesicle (Forzan *et al.*, 2004; Hassan *et al.*, 2001; Hassan & Roy, 1999; Noad & Roy, 2009). However, recent studies with BTV-1 indicate that virion entry into BHK cells may also be mediated by macropinocytosis and not by clathrin dependent pathway (Gold *et al.*, 2010). During the entry process, both VP2 and VP5

are lost from the surface of the core and the core particle is released into the host cell cytoplasm.

All reoviruses replicate in the cytoplasm of the infected cell, where the viral dsRNA genome is protected from the host-cell anti-viral responses by the core particle structure (Jacobs & Langland, 1996; Mertens & Diprose, 2004). Therefore the dsRNA and all necessary enzymes including the helicase, polymerase and capping enzymes are also contained within the viral core. Following the removal of outer capsid proteins the core becomes transcriptionally active (Mertens & Diprose, 2004; Van Dijk & Huismans, 1988) and the viral helicase is thought to unwind the strands of the dsRNA ahead of the viral polymerase. During the initial transcription process, the viral polymerase (VP1) simultaneously and repeatedly synthesises only positive sense RNAs (mRNAs) from the ten genomes segment (Urakawa *et al.*, 1989). The nascent mRNAs are then capped and methylated by the viral capping enzyme (VP4) (Ramadevi *et al.*, 1998; Sutton *et al.*, 2007) then extruded into the cytoplasm from the core at the five-fold axes (Figure 1.5) (Diprose *et al.*, 2001; Grimes *et al.*, 1998; Mertens & Diprose, 2004). The substrates for the transcription, e.g. nucleotide triphosphates (NTPs) and its by-products, are transported in and out of the core by alternative channels (Diprose *et al.*, 2001).

The ten capped messenger or positive-sense RNAs synthesized by the parental virus core act as translation templates for the synthesis of viral proteins. mRNA of all ten genome segments are synthesised by the parental or progeny virus cores that are located in or associated with the viral inclusion bodies (VIB). The VIB contain all of the virus-structural proteins, viral RNAs and NS2, as well as the ssRNA binding phosphoprotein (Modrof *et al.*, 2005; Thomas *et al.*, 1990). The progeny virus particles assemble within the VIB, using only one viral mRNA of each genome segment associated with each

nascent inner capsid. The encapsidation process within the VP3 shell involves interactions with the helicase (Stauber *et al.*, 1997), the ssRNA binding NS2 protein (Kar *et al.*, 2007), the VP1 and VP4 proteins (Nason *et al.*, 2004). These nascent particles are thought to mature via a process that involves of the core surface protein VP7(T13). VP1 then synthesizes the negative strand RNA, using the encapsidated mRNA transcripts as templates, to produce dsRNA within the progeny cores (Boyce *et al.*, 2004; Mertens & Diprose, 2004).

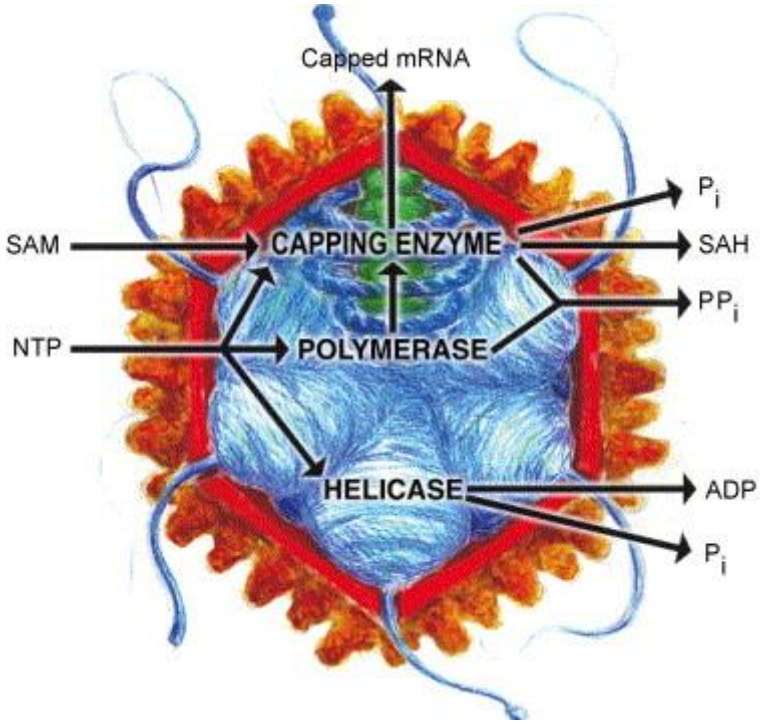


Figure 1.5: Graphical representation of a transcriptionally active core-particle of BTV or AHSV.
Each individual genome segment is transcribed simultaneously by transcription complexes located at the five-fold axes and the capped mRNA is extruded through pores at these axes. Transcription substrates and by-products are transported via alternative pores (Mertens & Diprose, 2004).

The outer capsid components (VP2 and VP5) are added as particles leave the VIBs and enter the host cell cytoplasm (Brookes *et al.*, 1993; Hyatt & Eaton, 1988). However the mechanism by which fully mature progeny virions are formed is not yet fully understood. Mature progeny virus particles are transported within the cytoplasm on microtubules involving VP2 / vimentin (intermediate filaments) interactions (Bhattacharya *et al.*, 2007). Intracellular transport of progeny virus particles is thought to be mediated by NS3, resulting in their movement to the cell surface where virus release occurs, again mediated by NS3 viroporin activity (Meiring *et al.*, 2009; Noad & Roy, 2009). However, recent studies with NS4 suggest that it may also play a role in cell-exit (Belhouchet *et al.*, 2011). Virus release can occur by budding, as seen in insect cells (Meiring *et al.*, 2009; Noad & Roy, 2009); by cell lysis (Schwartz-Cornil *et al.*, 2008; Stoltz *et al.*, 1996); or by direct penetration of the cell membrane (Fu, 1995). All three forms of virus release have been observed at different stages of BTV infection of mammalian cells (Fu, 1995). Once released, the progeny particles are able to infect new cells or 'superinfect' the same cell, leading to further amplification of the infection and replication process (Hyatt *et al.*, 1989).

1.7: The disease

1.7.1: Geographical distribution and major outbreaks of AHSV

African horse sickness is currently endemic in Sub-Saharan Africa in a vast area stretching across the central part of African continent - from Gambia and Senegal in the west to Ethiopia in the east, and extending to South Africa in the south. Here all nine serotypes are capable of causing the disease. The enzootic distribution of the disease to the north is restricted by Sahara desert, which appears to provide a geographical barrier

which, till now, has prevented establishment of the virus in northern Africa and beyond (Mellor, 1993; Mellor & Hamblin, 2004; Mellor & Mertens, 2008). AHS may also be endemic outside Africa, in Arabian Peninsula, where AHSV-9 was confirmed in 1998 (Anderson *et al.*, 1989; Mellor *et al.*, 1990b; Sailleau *et al.*, 2000). However a study using sentinel animals, conducted between 1992 and 1995 in Yemen did not detect AHSV in this country (Al-Afaleq *et al.*, 1998).

Major epidemic outbreaks of AHS, caused by all nine serotypes of the virus, occur regularly in north-eastern provinces in South Africa (Coetzer & Guthrie, 2004). They are thought to be linked to the warm El Niño/Southern Oscillation, a periodic weather related fluctuation in the Pacific Ocean which causes climatic variability, every ten to fifteen years (Baylis *et al.*, 1999). In the last few decades, the frequency of annual AHSV outbreaks has declined in comparison with historical data. This is thought to be related to elimination of large populations of zebra – the primary wildlife reservoir-host of AHSV (Barnard, 1998) (see Section 1.7.2) and the introduction of vaccination (Mellor & Hamblin, 2004) (see section 1.9). Recently, however, the situation has been changing with serotypes of the virus known to circulate sporadically (AHSV 5 and AHSV 9) now becoming dominant in some parts of the country (von Teichman *et al.*, 2010) and repeated outbreaks taking place in the surveillance zone around the Cape West zone (an AHSV-free zone) (ProMED-mail, 2007c; Venter *et al.*, 2006). Last year, an AHSV outbreak in South Africa was reported to be exceptional "killing more than 400 horses a day" countrywide (ProMED-mail, 2011).

Until relatively recently, outbreaks of AHS were confined to sub-Saharan Africa. However in 1959 AHSV-9 appeared in Middle East and spread throughout Saudi Arabia, Syria, Lebanon, Jordan, Iraq, Turkey, Cyprus, Iran and Afghanistan as far as Pakistan and India (Howell, 1960). Despite efforts to restrict spread of the disease,

including massive vaccination and vector control programmes, the outbreak only came to the end in 1961, after virtual extinction of susceptible hosts in the region (over 300 000 equids died as a result of the outbreak) (Howell, 1960). The same serotype of the virus (AHSV-9) caused another outbreak in North Africa in 1965 which also briefly affected southern Spain (Anderson *et al.*, 1989; Lubroth, 1988; Mellor *et al.*, 1990b). This outbreak was thought to have been caused by transportation of infected donkeys across the Sahara (Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004; Wilson *et al.*, 2009a).

The most recent European outbreak of AHS was caused by importation of AHSV-4 in infected zebra, from Namibia to a Safari Park near Madrid in 1987 (Lubroth, 1988; Rodriguez *et al.*, 1992; Sanchez-Vizcaino, 2004). Before the outbreak ended in 1991, it had spread to Portugal and Morocco (Mellor & Hamblin, 2004; Portas *et al.*, 1999). The virus successfully persisted in the Iberian Peninsula for a number of years before being eradicated, suggesting that it can be transmitted and could survive (indicating suitable vectors) in many areas beyond its current distribution (Mellor & Hamblin, 2004; Wilson *et al.*, 2009a).

Eight serotypes of AHSV (AHSV-1 to AHSV-8) are thought to be circulating in southern Africa while AHSV-9 is believed to predominantly affect horses in central and west Africa. However, this established pattern recently began to change. In 2007 an outbreak of AHSV-2 affecting equids was detected in Nigeria (Fasina *et al.*, 2008; Kazeem *et al.*, 2008; OIE, 2007; ProMED-mail, 2007b) and Senegal (OIE, 2007; ProMED-mail, 2007a), making it the first report of this serotype in the western part of central Africa. Then by 2008, the AHSV-2 outbreak had spread to the east causing casualties in equids in Ethiopia, (OIE, 2008; ProMED-mail, 2008). Later in 2010, AHSV-2 was also reported in Ghana (OIE, 2010; ProMED-mail, 2010). In 2007, two

more serotypes were detected: AHSV-4 in Kenya and AHSV-7 in Senegal (Maan *et al.*, 2011a; Mertens *et al.*, 2009). In 2010 an outbreak involving five different serotypes of AHSV (AHSV-2, 4, 6, 8 and 9) took place in Ethiopia (see Chapter 6). Although, this was the first incidence of simultaneous detection of circulation of different AHSV on such scale in the northern hemisphere, outbreaks involving multiple serotypes of AHSV (AHSV-1, 2, 4, 5 and 6) have been previously reported in South Africa (ProMED-mail, 2009). A serological study conducted in selected districts in Ethiopia at the time of the AHSV outbreak (November 2009 and February 2010) showed high level of AHSV prevalence in donkeys, mules and horses (Bitew *et al.*, 2011).

1.7.2: AHSV host species

Equid species and crossbreeds (e.g. horses, donkeys, asses and zebras) are the main vertebrate hosts of AHSV. Horses are the most susceptible species, showing the most severe clinical signs of AHS. Horses also have the highest mortality rate of the disease (70-95%) in comparison to mules (50-70%) and donkeys, which rarely displays clinical signs (<10% mortality rate). AHSV in zebra is asymptomatic (Coetzer & Guthrie, 2004).

The first reports of AHSV are dated after the introduction of horses into southern and eastern parts of Africa (Henning, 1949) and outbreaks of the disease have occurred regularly in sub-Saharan Africa ever-since. Horses are regarded as an indicator species for the disease, while zebra are the most resistant and are believed to be the natural vertebrate reservoir-host for the virus, playing an important role in its persistence in Africa (Barnard *et al.*, 1994; Lord *et al.*, 1997). Indeed, historically, endemic transmission of AHSV was restricted to areas containing zebra species (Barnard, 1998).

Despite the important role that zebra are thought to play in transmission and persistence of AHSV in the African continent, a number of epizootics have occurred in areas beyond their normal regional distribution (see Section 1.7.1). Currently, large numbers of equids, including horses, are kept in countries of central and northern Africa (Figure 1.5).

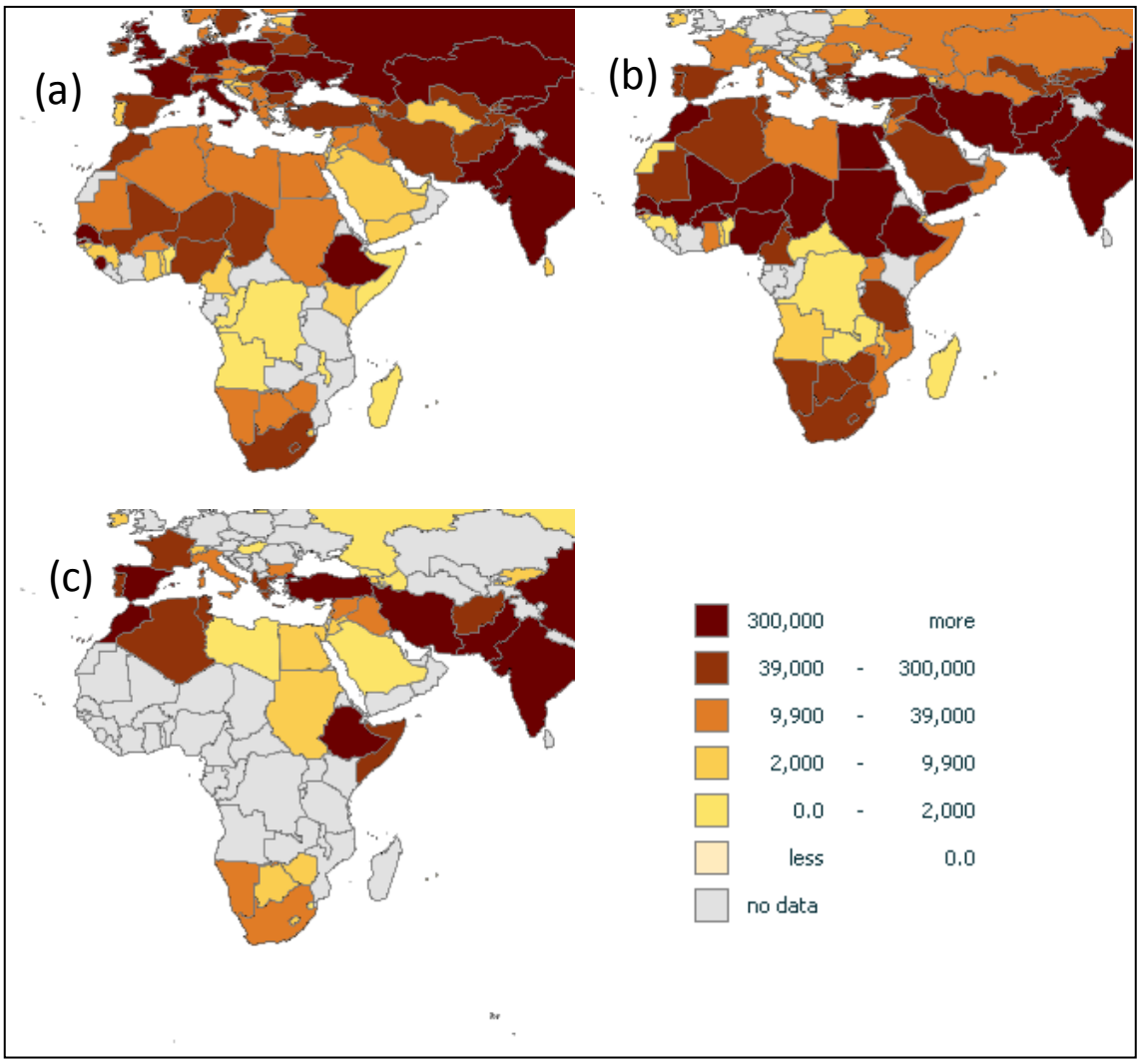


Figure 1.5: Distribution of equids in Africa and neighbouring regions.
Respective numbers of horses (a), asses (b) and donkeys (c) in different countries in 2007 are indicated by colour gradient. Maps were created using Global Livestock Production and Health Atlas of the Food and Agriculture Organization of the United Nations (FAO) and accessed at http://kids.fao.org/glipha/.

Apart from equids, certain large carnivores (including domesticated dogs) can also be infected by AHSV, as a result of eating infected meat (Van Rensburg *et al.*, 1981). They can develop clinical signs of the disease, usually a pulmonary form, and may die as a result of the infection. Most of free-ranging African carnivores (cheetahs, lions, African wild dogs, Jackals, hyenas, large-spotted genets) as well as domestic dogs were found to carry antibodies to five different serotypes of AHSV, mainly of serotypes 4 and 7 (Alexander *et al.*, 1995). However, another study of fee living zebra in Tanzania revealed different serotypes of AHSV (2, 5, and 9) to be predominant in this area (Hamblin *et al.*, 1990a). Transmission of AHSV from or between carnivores in the wild has not been reported and the significance if any of their overall role in the transmission cycle of AHSV is unproven (Mellor & Hamblin, 2004).

1.7.3: AHSV vector species

AHSV is primarily transmitted via the bites of adult females of certain *Culicoides* species – biting midges. Female midges feed on the blood of a vertebrate host, to provide a protein source for egg production, and can become infected or transmit the virus during this process (Meiswinkel *et al.*, 2004). The main species of *Culicoides* involved in transmission of AHSV is *C. imicola* (Bouayoune et al., 1998; Meiswinkel, 1998; Meiswinkel et al., 2004; Mellor, 1993b), which is common throughout Africa and South East Asia. *C.imicola* has also been identified in Mediterranean Europe, including southern Spain (Mellor *et al.*, 1983), mainland Greece and European Turkey (Purse *et al.*, 2005) and Southern France.

The current distribution of *C. imicola* appears to be limited by temperature. However, this vector species can be spread over large distances, over land and sea as aerial

plankton (Purse *et al.*, 2005). Predictions made on the basis of an increase in the global mean temperature of 2°C, suggest a further spread northwards into Europe, possibly as far as coastal regions of Albania, Bosnia and Croatia, as well as a wider distribution in southern France and parts of northern Italy (Purse *et al.*, 2005; Wittman *et al.*, 2001).

C. imicola breeds in damp, organically enriched soil and can account for more than 90% of *Culicoides* collected in light-traps in locations where AHSV is endemic, although it is less common in cooler highland areas of South Africa (Meiswinkel *et al.*, 2004; Venter *et al.*, 2000a). The widespread belief that stabling animals can reduce infection with AHSV is supported by the reluctance of adult *C. imicola* to enter enclosed buildings (Wilson *et al.*, 2009a). However, a second field vector of AHSV was identified in Africa, *C. bolitinos* (Meiswinkel & Paweska, 2003; Venter *et al.*, 2000a), which appears to be less reluctant to enter animal housing (Meiswinkel *et al.*, 2000; Wilson *et al.*, 2009a). This *Culicoides* species is also widely distributed in southern Africa and is particularly common in highlands areas, where *C. imicola* is rare.

During the African horse sickness outbreak in Spain and Portugal (1987-1990) AHSV was isolated from mixed pools of *Culicoides* containing almost exclusively *C. obsoletus* and *C. pulicaris.* This indicates that these species could have been involved in transmission of the virus (Mellor *et al.*, 1990a). *C. obsoletus* and *C. pulicaris* are considered to be the most common *Culicoides* species across the whole central and northern Europe (Jennings & Mellor, 1988; Mellor & Hamblin, 2004) and are able to transmit BTV (Carpenter *et al.*, 2008; Carpenter *et al.*, 2009; Wilson *et al.*, 2009a).

Under laboratory condition *C. sonorensis*, the North American vector of BTV, can be infected and can transmit AHSV (Boorman *et al.*, 1975; Carpenter *et al.*, 2011; Mellor

& Hamblin, 2004; Wittmann *et al.*, 2002). This further supports the hypothesis that the effective vectors of BTV can also transmit AHSV.

1.7.4: AHSV transmission

For AHSV transmission to occur, the virus must be present in the peripheral blood vessels or in the skin of the infected host, making itself accessible to adult *Culicoides,* which act as vectors, while they feed on the host (Darpel *et al.*, 2012). After ingestion, the virus infects and replicates in the cells lining the insect's mid-gut. The progeny virus-particles, which are released into the haemoceol, are then disseminated within the insect and can infect the salivary glands where they undergo a second round of replication. The progeny virus particles are subsequently released into the lumen of the salivary gland ducts and can be transmitted to susceptible hosts during subsequent blood meals (Figure 1.6) (Mellor & Mertens, 2008; Wilson *et al.*, 2009a).

There are two incubation periods during the AHSV transmission cycle (Figure 1.6). These include an 'intrinsic' incubation period in the mammalian host, which is defined as the period between infection of the host, and the appearance of virus in its peripheral blood and skin. The second 'extrinsic' incubation period in the insect vector, is the period between ingestion of the virus by the insect and the appearance of the virus in its saliva, making it capable of infecting a susceptible vertebrate host. The duration of the extrinsic incubation period is dependent on ambient temperature and is reduced if the vector-insect is held for part or all of the time under warmer conditions (studies conducted at a temperature range of 15-30°C for AHSV-4) (Mellor & Mertens, 2008; Wilson *et al.*, 2009a; Wittmann *et al.*, 2002).

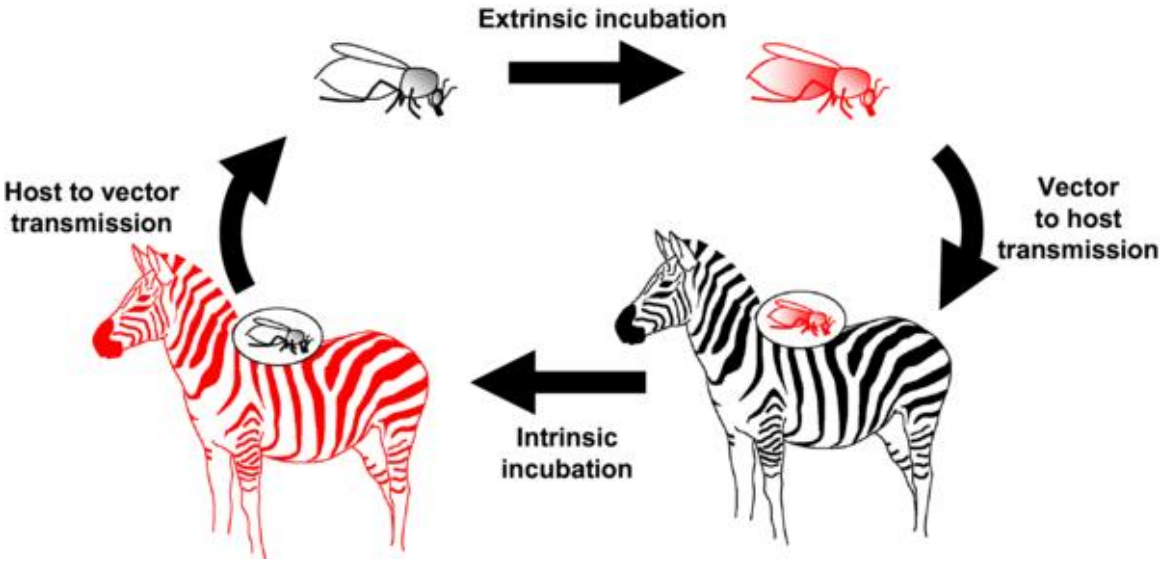


Figure 1.6: Transmission cycle of AHSV.
(Wilson *et al.*, 2009a).

Temperature can also affect transmission by influencing overall vector competence, survival and blood feeding intervals (Mellor & Mertens, 2008; Wellby *et al.*, 1996; Wilson *et al.*, 2009a; Wittmann *et al.*, 2002). Generally, vector insects take a blood meal more frequently at higher temperatures and the rate of virogenesis within the vector (which is dependent on virus derived enzymes such as VP1(Pol)) is increased, leading to a higher probability and frequency of transmission (Mellor, 1990). However, the lifespan of the insect vector may be shortened by increased environmental temperature, which could reduce the period and the number of occasions when the virus can be transmitted (Mellor, 1990). At lower temperatures, virogenesis slows and below 10-15 degrees it may even stop, but the lifespan of the vector may be increased (Mellor *et al.*, 1998).

1.7.5: Clinical signs of AHS

Following infection, AHSV replicates initially in the regional lymph nodes of the vertebrate host, from where progeny virus spreads throughout the body with blood circulation (primary viraemia), infecting secondary organs (lungs, spleen, other lymphoid tissues, pharynx and certain endothelial cells) (Burrage & Laegreid, 1994; Coetzer & Erasmus, 1994). Viral replication in these organs results in a secondary viraemia, the duration and titer of which depends on different factors, including host species, virulence of the strain and host susceptibility and current immune status (Coetzer & Guthrie, 2004). The viraemia caused by AHSV in horses (as measured by the detection of infectious virus) is usually short (4-8 days) and reaches a peak titer of up to $10^{5.0}$ $TCID_{50}$ ml^{-1}. However, donkeys and zebras typically develop a lower viraemia of $10^{2.5}$ $TCID_{50}$ ml^{-1}, although this usually lasts significantly longer (up to 28 days) (Coetzer & Erasmus, 1994; Hamblin *et al.*, 1998; Mellor & Mertens, 2008).

Like BTV, AHSV associates with blood cells during viraemia (Henning, 1956; Theiler, 1921), due to the hemagglutination activity of the outer capsid protein VP2 (Cowley & Gorman, 1987; Eaton & Crameri, 1989; Hassan & Roy, 1999). The attachment of virus particles to erythrocytes, where they may be protected from antibody mediated clearance, helps to explain the relatively long lived viraemia that has been detected by RT-PCR assays (up to 222 days in BTV infected sheep and up to 97 days in AHSV horses infected) (Bonneau *et al.*, 2002; Brewer & Maclachlan, 1992; Quan *et al.*, 2008). However, possibly due to the co-circulation of neutralising antibodies, these attached particles are thought to be non-infectious during the later stage of viraemia.

Oedema, effusion and hemorrhage are the most obvious features of AHSV pathology, which are thought to be caused by a loss of the endothelial cell barrier particularly in

the smaller blood vessels and capillaries (Burrage & Laegreid, 1994; Skowronek *et al.*, 1995).

Recent studies of the tissue and cell tropism of AHSV, conducted using a large number of animals, experimentally and naturally infected with all nine serotypes of the virus, confirmed that heart and lungs, followed by spleen are major tissue targets of the virus regardless of serotype or strain (Clift & Penrith, 2010). In BTV infected sheep or cows, a significant level of virus has been detected in fibroblasts, the endothelium of blood capillaries and cellular components of the hosts immune system, in the skin of the infected animal (Darpel, 2007). Since the skin is the largest single organ of the body and is involved as both the route of infection for the host and the route of transmission to the insect vector, it is thought to play an important role in the overall productivity and transmission cycle of BTV and the related orbiviruses (Darpel *et al.*, 2012).

AHS can be classified into four 'forms', according to the severity and type of clinical signs. These include pulmonary (acute), cardiac (subacute), mixed and febrile (horse sickness fever) forms (Coetzer & Erasmus, 1994). In horses, the clinical manifestation of the disease depend on the strain of virus causing the infection and the type of the host (Laegreid *et al.*, 1993). The most common finding in clinical cases of AHS is localized oedema of the lungs and/or subcutaneous and intramuscular tissues, effusion and hemorrhage. These are believed to result from replication of the virus in the vascular endothelium, leading to increased permeability of capillaries and larger blood vessels (Stoltz *et al.*, 1996).

The pulmonary (acute) form of AHS is the most severe and its progression is very rapid. An incubation period of three to five days is followed by a sudden onset of fever and soon after by respiratory distress. The head and neck of the horse may be extended

and severe sweating may occur. Terminally, frothy fluid may be discharged from the nares, and death can occur only a few hours after the first appearance of clinical signs. Prognosis for a horse suffering this form of the disease is very low and case fatality rates can often exceed 95%. This form of the disease is also seen most commonly in dogs (Coetzer & Erasmus, 1994). AHSV antigens were detected mainly in the cardio-vascular and lymphatic systems of experimentally infected horses, with this form of the disease (Wohlsein *et al.*, 1998).

The cardiac (sub-acute) form of AHS has an incubation period (7-14 days) and duration of illness (4-8 days) that are longer than in the pulmonary form. Clinical signs include subcutaneous oedema, particularly of the neck and head (characteristically of the supraorbital fossae), which may progress to the whole body. Petechial haemorrhages of the conjunctiva may also appear. Mortality rate can exceed 50% and death is usually due to cardiac failure (Coetzer & Erasmus, 1994).

The mixed from of AHS is the most common. It represents a combination of cardiac and pulmonary forms in which either form may predominate and the case fatality rate is usually high at 50-90% (Coetzer & Erasmus, 1994).

The ‘febrile’ form of the disease is considered to be the least severe and may be observed in animals with some level of immunity, or as a result of infection with less virulent (e.g. modified live vaccine) strains of virus. Only relatively mild forms of the disease are also seen in the African donkey and zebra. During the course of the disease a mild to moderate increase in temperature is observed as well as anorexia, and conjunctivitis. The symptoms of the disease are short-lived and recovery rapid and absolute (Coetzer & Erasmus, 1994). In experimentally infected horses the virus antigen is mainly concentrated in the spleen (Wohlsein *et al.*, 1998).

1.7.6: The economic importance of AHS

The economic importance of African horse sickness is mainly due to its severity and high mortality in naïve horse populations, as illustrated the devastating history of AHSV outbreaks outside its endemic zone. However, it can also cause losses due to trade restrictions and limitations on the movement of animals and animal products (de Vos *et al.*, 2012). Between 1959-1961 an outbreak caused by AHSV-9 (see Section 1.7.1) killed over 300,000 equids (Howell, 1960) almost bringing to extinction hosts susceptible to the virus in the outbreak area. Over 1000 equids also died or were destroyed in Spain alone, as a result of the 1966 AHSV-9 outbreak (Lubroth, 1988). Even more horses (about 1500) died or were destroyed over four years (1987-1990) during the AHSV-4 outbreak in Spain (Sanchez-Vizcaino, 2004).

AHSV outbreaks, have huge financial consequences in non-endemic areas, not only due to the relatively high cost of the individual animals involved, but also due to knock on effects on international trade, movement of animals or animal products (equine semen, ova and embryos), the costs of surveillance, eradication and vaccination campaigns. The total cost of the eradication of the AHSV-4 outbreak episode in Portugal during 1998 (including compensations to the horse owners, cost of vaccines and the administrative costs of vaccination) was estimated to be around US $2 million, but this does not include the financial consequences of prohibition of internal and international transportation of equids (Portas *et al.*, 1999). Economic losses due to a hypothetical AHSV outbreak in the Netherlands were recently estimated at 272-516 million Euros (de Vos *et al.*, 2012). Taking onto account the estimated £7 billion annual value of the horse industry in the UK (DEFRA, 2010), losses due to an AHSV outbreak in this country would also be very severe (DEFRA, 2008).

AHSV could be introduced to non-endemic areas as a consequence of (a) legal or illegal trade in equids, (b) legal or illegal trade of equid products, (c) illegal use of a live AHSV vaccine, or (d) entry of infectious vector insect(s). Movement and importation of equid species into countries of the European Union is highly regulated (EEC, 1990) and therefore the risk of this route of AHSV introduction to the UK (DEFRA, 2008) and the Netherlands is considered very low (de Vos *et al.*, 2012). However, it is impossible to quantify the likelihood of virus introduction through other, as yet, unspecified routes (de Vos *et al.*, 2012; DEFRA, 2008).

According to data from the Food and Agriculture Organization of the United Nations (FAO), large numbers of horses and asses are reared in the countries of central Africa, particularly in Senegal (in the west) and Ethiopia (in the east) (Figure 1.5). In Ethiopia, horses, donkeys and mules are used as working animals and, in many cases, receive little care (Bitew *et al.*, 2011). The large number of equids susceptible to AHSV in Ethiopia, including those which rarely display clinical signs of disease, and the poor veterinary care that they receive, may facilitate transmission of AHS into new areas (see Section 1.7.1 and Chapter 6). The recent detection of multiple serotypes of AHSV in central Africa, particularly in Ethiopia (Aklilu *et al.*, 2012), suggests that the virus could spread from these areas to cause outbreaks in other geographic regions, including Europe and the Arabian Peninsula.

The route of BTV-8 introduction to Europe in 2006 has not yet been identified (Carpenter *et al.*, 2009), however the virus overwintered and spread over large areas causing substantial losses to the agriculture industry of several countries over a number of years (Carpenter *et al.*, 2009; de Koeijer *et al.*, 2011). With progressing climate change, the distribution of vector born disease is also likely to change (MacLachlan &

Guthrie, 2010; Purse *et al.*, 2008; Purse *et al.*, 2005), suggesting that AHSV could once again emerge from Africa .

Phylogenetic data indicated that the BTV-8 strain, which was introduced to Europe in 2006 (via an unknown mechanism) was closely related to strains of this serotype circulating in Sub-Saharan Africa (Nigeria) (Maan *et al.*, 2008). Also, Schmallenberg virus (an orthobunyavirus) which emerged in northern Europe during 2011, is closely related to Shamonda virus, which originated from Nigeria (Goller *et al.*, 2012; Saeed *et al.*, 2001). Based on the recent change in distribution of arboviruses in Europe it is considered likely that other viruses originating from sub-Saharan Africa, potentially including AHSV, could arrive in northern Europe.

1.8: Diagnosis of AHSV

Initial diagnosis of African horse sickness is based on observation of clinical signs combined with epidemiological information. However, laboratory conformation of the etiological agent is required to confirm the causative agent (OIE, 2012). Despite being lengthy (taking weeks) and labour intensive, serological methods using virus or serum neutralization tests (VNT or SNT) remain as important ‘gold standard’ methods for the detection and identification of AHSV, by serotyping the virus (Howell, 1962; McIntosh, 1958). However, these methods depend upon the availability of reference antisera, and/or reference virus strains, the serotype-specificity of which is known. These important reagents are not usually available outside specific reference laboratories, limiting accessibility to serological typing assays. More recently assays to detect AHSV RNA have been developed and validated, that depend on synthetic oligonucleotides for their specificity, making diagnostic systems more readily available.

1.8.1: Identification of AHSV antigens

1.8.1.1: Virus isolation

Isolation of AHSV has traditionally formed an important part of diagnosis and provides material for virus strain/serotype identification. The virus can be isolated from whole blood, but due to rapid progression of the disease and death, isolation from homogenised tissues such as spleen, lung and lymph nodes, collected at post mortem examination, can also be performed. Two *in vivo* systems are described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals issued by OIE: intravenous inoculation of embryonated hens' eggs and intra-cerebral inoculation of suckling mice (Mellor & Hamblin, 2004; OIE, 2012). For *in vitro* isolation, tissue culture such us baby hamster kidney (BHK), monkey stable (MS), African green monkey kidney (Vero), and insect derived cells (either KC cells, from the orbivirus insect-vector species *Culicoides sonorensis* (=*variipennis*), or C6/36 cells, from the mosquito *Aedes albopictus* cells) have been used (Mellor & Hamblin, 2004; OIE, 2012). The insect cell lines appear to be very tolerant to high concentration of blood in their culture medium (possibly reflecting their derivation from haematophagus vector-species) facilitating virus isolation. Unlike most mammalian cells, the insect cell systems infected with AHSV (or BTV) do not exhibit cytopathic effects (CPE) and further methods are therefore required to confirm virus replication, involving viral antigen or RNA detection, or transfer to a mammalian cell-line that does show CPE (OIE, 2012).

1.8.1.2: Serological methods

Historically, after virus isolation, the AHSV antigen was confirmed using a complement fixation (CF) (McIntosh, 1956). Despite still being recommended by OIE

(OIE, 2012), this method has been largely replaced by other serological techniques, including enzyme-linked immunosorbent-assays (ELISA) and RNA based assays (see Section 1.8.1.3). A number of different ELISA tests have been developed for detection of AHSV antigens (Du Plessis *et al.*, 1990; Hamblin *et al.*, 1991; Laviada *et al.*, 1992) but they are labour intensive and rely on a concentrated source of antigen. For this reason, they are usually used to characterise tissue culture propagated virus and therefore, because of the time taken to adopt the virus to cell culture, can be slow in identification of field strains.

The identification of AHSV serotype using virus neutralization tests (VNT) usually depend on isolation and adaptation of the virus to growth in cell culture (Howell, 1962). A dilution series of the virus is then treated with a standard amount of 'reference' neutralising antisera for each of the different serotypes, prior to infection of cell cultures. A reduction of virus titre of at least 100 fold, compared to pre-immune control sera, is usually regarded as evidence of a 'type' specific reaction (OIE, 2012).

Despite the development on molecular tests for AHSV RNA that will also identify serotype (see section 1.8.1.3 and Chapter 4) VNT assays are still regarded as a "gold standard". These serotyping assays, however, are labour intensive and time consuming (5-7 days for the virus titration), may depend on prior virus isolation (VNT), and are dependent on the availability of reference virus strains and reference antisera, which are highly characterised, may require special disease secure facilities for handling and may therefore be difficult to obtain.

1.8.1.3: RNA detection and characterisation methods

The genetic material (dsRNA) of AHSV can be detected and identified by a number of currently available methods, including (a) molecular probes, (b) conventional and real-time RT-PCRs and (c) sequence comparisons.

The majority of published group (virus-species) specific assays for detection of AHSV are conventional PCRs targeting one or more of the more conserved segments of the virus genome. The amplification products of these assays are visualized by AGE and their specificity is assessed by estimations of the size and relative amounts of the amplification product. Different conserved AHSV genome segments have been targeted in these assays including Seg-3 (Aradaib, 2009; Aradaib *et al.*, 2006; Sakamoto *et al.*, 1994b), Seg-8 (Moulay *et al.*, 1995; Sailleau *et al.*, 1997a; Zientara *et al.*, 1993; Zientara *et al.*, 1994; Zientara *et al.*, 1998; Zientara *et al.*, 1995b), Seg-5 (Mizukoshi *et al.*, 1994), Seg-9 (Bremer & Viljoen, 1998; Stone-Marschat *et al.*, 1994), and Seg-10 (Zientara *et al.*, 1995b). All of these methods have been shown to detect AHSV but may not have been validated with a large number of samples of all serotypes of AHSV.

Sensitivity of these assays was heightened by the introduction of a second PCR amplification step - a "nested" step (Aradaib, 2009; Moulay *et al.*, 1995). However, despite high correlation in the level of detection of AHSV, in comparison to virus isolation, some strains with low virus load still could not be identified (Sailleau *et al.*, 1997a). In addition, introduction of this step makes the RT-PCR detection process labour-intensive and increases both costs and the possibility of sample contamination (false positives).

The analysis of conventional RT-PCR assays by AGE to detect the amplicons that are generated requires that the reaction tubes are opened post-amplification, with the

potential that the large amounts of cDNA generated could lead to contamination and false positive reactions in subsequent assays. However, the chemistry of real-time RT-PCR assays (as further discussed in Chapter 2 Section 2.8) allows precise detection of target amplification in a close tube format, which significantly improves sample throughput and reduces contamination risk. Several real-time RT-PCR assays for the detection of different genome segments of AHSV have been described including Seg-7 (Aguero *et al.*, 2008; Fernandez-Pinero *et al.*, 2009), Seg-5 (Rodriguez-Sanchez *et al.*, 2008), Seg-8 (Monaco *et al.*, 2011) and both Seg-7 and Seg-9 (Quan *et al.*, 2010).

In general, the analytical sensitivity (detection of low concentrations of the target sequence) of these real-time assays is better than that of the conventional RT-PCR assays and they all were shown to be able to detect different serotypes of AHSV. However diagnostic specificity (ability of the assays to detect different isolates) of these assays can only be assessed by testing wide range of different AHSV isolates from various geographical origin. The diagnostic sensitivity of the published assays was primarily assessed on the basis of the nine South African reference strains of AHSV and excluded testing of any field strains. Indeed, *in silico* analyses, based on sequence data from southern African isolates of AHSV (Quan *et al.*, 2010), have shown nucleotide discrepancies between the assays target regions in the viral RNAs and the oligonucleotide components of the published assays (Aguero *et al.*, 2008; Fernandez-Pinero *et al.*, 2009; Rodriguez-Sanchez *et al.*, 2008). New, extensively validated serogroup specific assays, detecting independently conserved genome segments 1 and 3 (encoding structural proteins 1 and 3) have been developed and are described in Chapter 4.

The detection of the AHSV genome in blood or tissue samples indicates that the animal being tested has recently been infected with AHSV, but it does not demonstrate that it is

still infectious. This quality of the method is of a particular importance when used for diagnosis during an outbreak situation or for screening animals and animal products for international trade and movement purposes, allowing rapid identification of infected animals. During the recent outbreaks of bluetongue in northern Europe, relatively crude inactivated vaccines were used with great success to eradicate the virus (Bartram *et al.*, 2011; Bréard *et al.*, 2011; Eschbaumer *et al.*, 2009; Moulin *et al.*, 2012). However these vaccines generate antibodies to all of the viral proteins, invalidating most serological diagnostic methods that could be used to distinguish vaccinated from infected animals (DIVA assays). Under these circumstances the long lived viraemia caused by infection (due to the viral haemaglutinin – see Section 1.7.5) means that recently infected animals can be identified by RT-PCR and these assays can therefore be used for surveillance purposes (Batten *et al.*, 2008a; Batten *et al.*, 2009).

The recent development of improved sequencing methods for the segmented dsRNA viruses which avoid the necessity for cDNA cloning (Maan *et al.*, 2007b; Potgieter *et al.*, 2009) have facilitated the full-length characterization of AHSV genome segments and of whole genomes (Oura *et al.*, 2011) (Chapters 3, 5 and 6). These developments have made it easier to correlate variations in Seg-2 sequences with the serological properties and serotype of different BTV and EHDV isolates (Anthony *et al.*, 2009c; Maan *et al.*, 2007a). Similarly, serotype dependant grouping in Seg-2 has been described for AHSV (Potgieter *et al.*, 2003).

The first molecular method described serotype specific differences in restriction fragment length polymorphism (RFLP) of a PCR product of genome segment 7 and 10 (Zientara *et al.*, 1995a; Zientara *et al.*, 1998), although due to genome segment reassortment, this cannot be regarded as a reliable technique for serotype identification. More recent sequencing data show that neither Seg-7 (encoding VP6/NS4) nor Seg-10

(encoding NS3/NS3a) group in a serotype specific manner (Anthony *et al.*, 2009a; Maan *et al.*, 2010b)(Chapter 3).

The development of more reliable Seg-2 based serotyping methods include (a) direct sequencing of Seg-2 followed by sequence comparison to Seg-2 sequences of established reference strains (Chapter 3, section 3.2.3.2), (b) conventional RT-PCR (Maan *et al.*, 2011a; Sailleau *et al.*, 2000), (c) and cross-hybridisation methods (Koekemoer *et al.*, 2000; Koekemoer & van Dijk, 2004). Method c was also developed into a real-time, adjacent hybridization probe method based on analysis of post PCR melting curves and their comparison to empirically established melting curves of the nine reference strains (Koekemoer, 2008). However, as Seg-2 is the most variable genome segment of AHSV the curve's dissociation characteristics for individual isolates within respective serotypes can vary, making the technique relatively unreliable. Methods a and b, particularly if used in combination have been shown to be very reliable, if labour intensive.

1.8.2: Identification of AHSV specific antibodies

Traditional serological methods for the detection and identification of AHSV group-specific antibodies include agar gel immuno-diffusion (AGID) (Verwoerd *et al.*, 1979) and complement fixation (CF) (McIntosh, 1956). The CF test is still recommended by OIE for identification of AHSV specific antibodies for international trade purposes (OIE, 2012). The development of ELISA's systems for detection of antibodies against AHSV overcomes some of the drawbacks of these traditional tests, including a lack of standardization and unsuitability for high throughput (Hamblin *et al.*, 1992; Hamblin *et al.*, 1990b; Kweon *et al.*, 2003; Laviada *et al.*, 1992; Laviada *et al.*, 1995; Maree & Paweska, 2005). These assays rely on the generation of antibodies to AHSV proteins

that are conserved between the different members of the virus species / serogroup, during infection of the vertebrate host, primarily targeting VP7, the major orbivirus-group-specific antigen (Huismans & Erasmus, 1981; Maree & Paweska, 2005; Peeters & Van der Spek, 2005). An indirect ELISA targeting non-structural protein NS3 of AHSV-4 has also been described (Laviada *et al.*, 1995).

ELISA tests to detect AHSV specific antibodies do not require virus isolation and are therefore much more rapid than antigen detection assays. However, since antibodies to AHSV are thought to be long-lived cELISA cannot be used to reliably distinguish 'older' from 'more recent' infections. However, most infected horses die during the course of AHS, before virus specific antibodies have fully developed. These assays systems therefore have only limited value for laboratory diagnosis of the disease, but can be used to assess the immunological status of animals for trade and, are recommended by OIE for this purpose (OIE, 2012).

Serotype specific neutralising antibodies against AHSV, which are generated during virus infection in the mammalian host, can be detected in serum neutralisation tests (SNT). These assays test the ability of the serum to neutralise the homologous virus type. This method can use a dilution series of the test antiserum, reacted with a known amount of a characterised reference virus stock (e.g. 100 $TCID_{50}$). The titre of the serum is determined by the dilution at which neutralisation still takes place (House *et al.*, 1990).

Alternatively, in virus neutralisation test, a known dilution of the test antiserum can be used (e.g. 1/100) to examine a dilution series of different viruses, to compare its ability to neutralise different serotypes. The homologous type will show a 100 fold greater reduction in titre compared to heterologous types (Mertens *et al.*, 1989b).

Sequential infection with one or more serotypes of BTV can lead to high levels of neutralising antibodies, not only to the two serotypes involved, but also showing cross-reactivity with other serotypes (Jeggo *et al.*, 1986). It can therefore be difficult to identify the individual serotypes that have infected an animal, in areas where multiple types are known to be circulating, by SNT.

1.9: Control of AHS and vaccination against AHSV

AHS was classified as a class A disease, by the Office International des Epizootics (O.I.E) (OIE, 2011) and remains a 'notifiable' disease within Europe (European Economic Committee Council Directive 82/894/EEC, on the notification of animal diseases within the Community (EEC, 1982)).

Apart from rest and good husbandry practices, there is no specific treatment for animals suffering clinical signs of AHS (Mellor & Hamblin, 2004). Recent study have shown that silencing of the AHSV VP7 gene with short interfering RNAs (siRNA) results in a decrease in infectious particle production, in comparison to an untreated control (Stassen *et al.*, 2007). This approach could be considered in the development of antiviral treatment. However, clinical sings of AHSV infection are progress very rapidly and appear when tissue damage had already occurred (Coetzer & Guthrie, 2004). This approach may not therefore be practical as an antiviral drug.

Taking into account that AHSV is non-contagious and the disease is passed primarily through the a bites of infected vectors (*Culicoides* spp.), control measures for AHS focus primarily on prevention of further virus spread and include: (a) movement restrictions of susceptible animals, (b) control of vector populations, (c) removal of infected animals as they could act as a source of further virus dissemination into insect

population, (d) modifications to husbandry practices which involve stabling of susceptible animals (e) introduction of surveillance and protection zones and (f) vaccination. The above control measures which prevent further spread of AHSV in endemic areas are guided by the Terrestrial Animal Health Code of OIE (OIE, 2011), while in the countries of European Union they are strictly regulated by the Council Directive 92/35/EEC (EEC, 1992).

Perhaps the most important measure to control AHS outbreaks, in both, endemic and non-endemic areas is vaccination. Currently, only live attenuated vaccines, generated by multiple passage of the virus in cell culture, are commercially available, from Onderstepoort Biological Products (OBP, a Onderstepoort, South Africa) (Coetzer & Guthrie, 2004). These are polyvalent live vaccines, formulated in two vials, containing respectively serotypes 1, 3, 4 (bottle 1) and 2, 6, 7, 8 (bottle 2), which are recommended to be administered three weeks apart. Serotypes 5 and 9 are not included in the vaccine formulation; AHSV-5 because of difficulties with efficient attenuation (Mellor and Hamblin, 2004) and 9 as historically it rarely caused disease in South Africa (Coetzer & Guthrie, 2004). However, serotypes 8 and 6, offer some cross-protection against serotype 5 and 9, respectively, in vaccinated animals (Coetzer & Guthrie, 2004; von Teichman *et al.*, 2010).

During the 1987-1990 AHSV-4 outbreak in the Iberian peninsula, a monovalent AHSV-4 vaccine, manufactured by OBP was used in Spain (Lubroth, 1988; Sanchez-Vizcaino, 2004) and Portugal (Portas *et al.*, 1999). The National Laboratory of Senegal also produces a monovalent live attenuated AHSV-9 vaccine which has been used in West Africa, where serotype 9 of AHSV is predominant (Oura *et al.*, 2011).

Despite obvious benefits from using the live attenuated vaccines for the protection of susceptible animals in the AHSV endemic areas, there are still concerns regarding their wider use. Vaccinated animals that can also become naturally infected with a field strain of the virus could potentially generate reassortant viruses (von Teichman & Smit, 2008). Although the naturally produced reassortant-viruses detected in this study were not pathogenic or lethal to horses, reversion to virulence or further reassortment events cannot be ruled out in subsequent passages (von Teichman & Smit, 2008).

There is body of evidence that reassortant field and vaccine strains of BTV can circulate in the field (Batten *et al.*, 2008b; Maan *et al.*, 2010b). Live attenuated vaccines can induce viraemia in vaccinated animals (von Teichman *et al.*, 2010; von Teichman & Smit, 2008) which could lead to "leaking" of the strain into the vector population (Veronesi *et al.*, 2010; Veronesi *et al.*, 2005). It has also been shown that AHSV *Culicoides* vectors can be orally infected with vaccine strains of AHSV (Paweska *et al.*, 2003). In addition, the live attenuated vaccines do not offer full protection against infection with a field strain of the virus (Portas *et al.*, 1999; von Teichman *et al.*, 2010) in some cases leading to subclinical infection in immunized horses (Weyer *et al.*, 2012). For these reasons the use of live attenuated vaccines is strictly regulated in the countries outside AHSV endemic zone, including those of European Union (EEC, 1992). Should an outbreak occur in an AHSV free area the type of the virus would be determined before any vaccination program is initiated. Therefore the rapid determination of the type of AHSV involved is necessary for implementation of control through vaccination.

Other vaccines which could protect horses against AHSV infection have also been described, including inactivated vaccines (Sanchez-Vizcaino, 2004) and subunit vaccines (MartinezTorrecuadrada *et al.*, 1996; Roy *et al.*, 1996; Roy & Sutton, 1998; Scanlen *et al.*, 2002; Wade-Evans *et al.*, 1997). As a result of their ease, speed and low

cost of production, it appears likely that an outbreak of AHS in Europe would lead to the rapid production of an inactivated vaccine of the appropriate serotype, generated using virus grown in mammalian cell cultures (similar to those developed for BTV-8 to combat the 2006 outbreak in northern Europe (Bréard *et al.*, 2011; Moulin *et al.*, 2012)). It has been shown that cDNA of the gene encoding outer-capsid protein VP2 can elicit neutralising antibodies and can protect from homologous AHSV challenge in horses (StoneMarschat *et al.*, 1996).

Recently, new approach involving AHSV antigen delivery via a recombinant pox virus, such as canarypox (El Garch *et al.*; Guthrie *et al.*, 2009) or modified vaccinia Ankara (Castillo-Olivares *et al.*, 2011; Chiam *et al.*, 2009) have been tested in horses and mice. Although effective in generating a neutralising antibody response, these vaccines are not yet readily available through commercial sources.

Modified vaccinia Ankara (MVA) undergoes minimal or no productive replication in mammalian cells (Sutter & Moss, 1992). This MVA strain has lost 15% of its genome, including genes determining host range and virulence, after multiple passages in chick embryo fibroblasts (Meyer *et al.*, 1991). The replication process of MVA is blocked after the pox virus early and late genes are expressed (Sutter & Moss, 1992) allowing induction of humoral and cellular immune responses to the products of inserted exogenous genes in the mammalian host. MVA has extended packaging flexibility which allows large amounts of foreign DNA to be integrated in the genome, without the loss of infectivity (Meyer *et al.*, 1991) making it a suitable vector for delivery of foreign antigens (Breathnach *et al.*, 2004; Chiam *et al.*, 2009). MVA has been used in this project to delivery AHSV VP2, and its development and evaluation is further explored in Chapter 7.

1.10: Project aims and objectives

AHSV exists as nine different (known) serotypes, each of which can cause a devastating disease of horses - AHS. Although the distribution of AHS is currently restricted to Sub-Saharan Africa (as discussed in Section 1.8.1) recent evidence of multiple outbreaks of the disease outside its usual endemic zones, and epidemiological data concerning the emergence of multiple strains of BTV in Europe (since 1998), suggest that the distribution of AHSV could rapidly change. This project therefore aims to investigate differences in the genetic makeup (genome sequence) of different virus isolates, to explore the evolutionary and serological relationships between serotypes, and to create a sequence database for molecular epidemiology studies. It is anticipated that this will provide information concerning the origins and spread of viruses involved in recent and future AHS epidemics, both inside and outside the current endemic zones.

AHSV serotyping methods available to diagnosticians, although usually effective, can take a long time to produce definitive results, potentially prolonging the time taken to design and implement effective control strategies. As a result of the BTV-8 and Schmallenberg outbreaks in northern Europe, the risk of a hypothetical outbreak of AHS outside its current endemic zone, is estimated as significant (see Section 1.7.6). The economic consequences, due to the value of the equine industry, would also be huge. Therefore, in order to respond more rapidly to an outbreak of the disease and to minimise economic losses, the aims project was to develop diagnostic systems (real-time RT-PCR assays) able to detect and identify serotype of AHSV.

Specific aims of the project include:

- Generation of full length, full genome sequence data for the nine ‘historical’ reference strains of AHSV, to provide an initial sequence database for AHSV.

This database would be used for AHSV genome comparisons, including typing of new AHSV isolate, and as a base to design new and novel diagnostic tests (Chapter 3).

- Comparisons of the nucleotide and amino acid sequences of the different genome segments / proteins of the nine reference strains, to estimate their genetic relatedness (Chapter 3).
- Different topotypes have been identified grouping BTV and EHDV isolates from different continents. This project will investigate the level of diversity and phylogenetic grouping of AHSV, to determine if it also exists as different 'topotypes', even though it is primarily restricted to one continent (Chapter 3).
- Development of group specific real-time RT-PCR based on the nucleotide sequences generated for the nine reference and other strains of AHSV. This would involve optimisation, quantification and validation and of the assay(s) using an extensive number of different isolates of AHSV, including field strains from different geographical origins. Sequence data of highly conserved AHSV genome segments would be compared and regions suitable for design of amplification primes identified (Chapter 4).
- Development of RT-PCR assays for identification of specific AHSV serotypes, including their optimisation and validation with the available AHSV isolates of each type. For this purpose Seg-2 sequence data from different isolates of different AHSV serotypes would be compared in order to identify type specific regions – indifferent within serotypes but variable between types. (Chapter 4).
- Identification, generation and characterisation of nine monotypic reference strains of AHSV, by molecular means, so that these strains and their sequence

data can be made available as a resource to the wider community of researchers and diagnosticians (Chapter 5).

- Application of the novel AHSV Seg-2 database for confirmation of AHSV serotype in field isolates of the virus. It has been previously shown that comparison of Seg-2 sequences to the sequence database of reference strains constitutes a valuable method for typing of new isolates. (Chapter 6).
- Investigation of the phylogenetic relationship of Seg-2 within different serotypes of AHSV. Greater understanding of intra-serotypic variation AHSV would facilitate molecular epidemiological studies of historical and future outbreaks of the disease. This data would also enrich the Seg-2 sequence database of AHSV (Chapter 6).
- Investigation if serological relationship between of the nine reference strains of AHSV reflects their phylogenetic relationships. Phylogenetic analyses of Seg-2 indicate close relationship between certain serotypes of the virus. For this project, recombinant MVA expressing AHSV VP2 will be used to generate VP2 specific antisera raised in equidae. These antisera will be used for serological investigations (Chapter 7).

Chapter 2: Materials and methods

2.1: Cell culture

All cell culture work was performed using aseptic techniques in a Class II safety cabinet. Mammalian and avian cells were cultured at 37°C in 5% CO_2 atmosphere while insect cells were grown at 28°C in normal atmosphere. All media were supplemented with 10% foetal bovine serum (FBS) (heat inactivated at 56°C for 30 minutes prior to use) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Insect cell media was additionally supplemented with Amphotericin B (4 µg/ml). Most of the cell lines were obtained from the Central Service Unit (CSU), IAH Pirbright or from Microbiological Services, IAH Compton, while the BHK TK^{-} cell line was kindly provided by Dr. Javier Castillo-Olivares. Cell lines used in this study and their respective culture medium are summarised in Table 2.1.

Once they had reached confluence, monolayers of mammalian and avian cells were sub-cultured by rinsing the cell sheet three times with Trypsin-EDTA solution (Sigma) followed by 5-7 minutes incubation at 37°C until the cells had detached from the surface of the culture vessel. Detached cells were re-suspended in 10 ml of growth medium and split 1 in 5 for propagation or further diluted in growth medium in preparation of seeding tissue culture plates. Insect KC cells were sub-cultured by vigorous shaking of the culture vessel prior to dividing cells 1 in 10.

Table 2.1: Summary of cell lines and culture medium required.

Cell line	Medium
BHK-21 cells (Baby hamster kidney cells)	DMEM
BHK TK$^-$ cells (Baby hamster kidney cells, thymidine kinase negative)	DMEM
Vero cells (African green monkey kidney cells)	RPMI
DF1 cells (chicken embryo fibroblasts – continuous cell line) (Himly *et al.*, 1998)	DMEM
CEF cells (chicken embryo fibroblasts – primary cell line)	DMEM
KC cells (C. variipennis [now C. sonorensis] embryos cells) (Wechsler & McHolland, 1988)	Schneider's insect medium

DMEM = Dulbeco's modified Eagle's media.
RPMI = Roswell Park Memorial Institute medium.

2.2: Viruses

All virus isolates used in this study were obtained from the Orbivirus Reference Collection (ORC), IAH Pirbright. The origin and passage history as well as nomenclature of viruses used are listed in Table 2.2. Further information concerning individual viruses is available on the IAH Orbivirus reference collection web-site: http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/AHSV-isolates.htm.

Table 2.2: List of AHSV isolates used in the study.

Virus serotype	Origin	ORC nomenclature	Virus serotype	Origin	ORC nomenclature
Field strains			Reference strains		
AHSV-2	Ethiopia	ETH2010/01	AHSV-1	RSA	RSArrah/01
AHSV-2	Ethiopia	ETH2010/20	AHSV-2	RSA	RSArrah/02
AHSV-2	Ethiopia	ETH2010/23	AHSV-3	RSA	RSArrah/03
AHSV-2	Ghana	GHA2010/01	AHSV-4	Spain	SPArrah/04
AHSV-2	Ghana	GHA2010/02	AHSV-5	RSA	RSArrah/05
AHSV-2	Ghana	GHA2010/03	AHSV-6	RSA	RSArrah/06
AHSV-2	Ghana	GHA2010/04	AHSV-7	Kenya	KENrrah/07
AHSV-2	Ghana	GHA2010/05	AHSV-8	RSA	RSArrah/08
AHSV-2	Senegal	SEN2007/01	AHSV-9	Pakistan	PAKrrah/09
AHSV-2	Senegal	SEN2007/02	Monotypic reference strains		
AHSV-2	Senegal	SEN2007/03	AHSV-1	RSA	RSArah1/03
AHSV-2	Senegal	SEN2007/04	AHSV-2	RSA	RSArah2/03
AHSV-2	Senegal	SEN2007/05	AHSV-3	RSA	RSArah3/03
AHSV-4	Ethiopia	ETH2010/03	AHSV-4	SPA	SPArah4/03
AHSV-4	Ethiopia	ETH2010/04	AHSV-5	RSA	RSArah5/03
AHSV-4	Ethiopia	ETH2010/05	AHSV-6	RSA	RSArah6/03
AHSV-4	Ethiopia	ETH2010/06	AHSV-7	Kenya	KENrah7/03
AHSV-4	Ethiopia	ETH2010/07	AHSV-8	RSA	RSArah8/03
AHSV-4	Kenya	KEN2007/01	AHSV-9	Pakistan	PAKrah9/03
AHSV-4	Spain	SPA1987/01	Vaccine strains		
AHSV-6	Ethiopia	ETH2010/19	AHSV-2+3	Ethiopia	ETH2010/02
AHSV-7	Senegal	SEN2007/06	AHSV-4	Ethiopia	ETH2010/08
AHSV-8	Ethiopia	ETH2010/10	AHSV-5+7	Ethiopia	ETH2010/09
AHSV-8	Ethiopia	ETH2010/11	AHSV-5+9	Ethiopia	ETH2010/18
AHSV-9	Ethiopia	ETH2010/14	AHSV-9	Senegal	SENvvv1/09
AHSV-9	Ethiopia	ETH2010/15			
AHSV-9	Ethiopia	ETH2010/16			
AHSV-9	Gambia	GAM2009/01			
AHSV-9	Gambia	GAM2009/02			
AHSV-9	Gambia	GAM2009/03			
AHSV-9	Senegal	SEN1998/01			
AHSV-9	Kenya	KEN2006/01			

2.3: Virus propagation

For this study, AHSV was propagated in BHK-21, Vero cells or KC cells, in 175 cm^2 flasks. The Growth medium was removed from 80-90% a confluent monolayer of mammalian cells (BHK-21 or Vero cells) and cells were infected with 200-500 ml of virus (10-200$TCID_{50}$ /ml), diluted in 5 ml of the appropriate maintenance medium (without FCS), containing antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The virus was allowed to absorb to the cells for 30 min at 37°C and maintenance medium was added up to 40-45 ml. The infected cells were incubated at 37°C in an atmosphere with 5% CO_2, and periodically examined until 70-100% cytopathic effect (CPE) was observed, usually after 24-72 h. The infected cells were then harvested, and stored at +4°C until required.

A new set of AHSV monotypic reference strains (Table 2.2), were stored after molecular characterisation, in the Orbivirus Reference Collection (ORC), the Pirbright Institute (PIR). For this purpose, the harvested virus was mixed at equal volumes with Park Davis Additive Medium (PDAM) or with Glycerol (1,2,3-Propanetriol) (Sigma), respectively and stored (as multiple samples) at -80°C.

An insect cell line derived from Culicoides sonorensis (KC cells) were also used for propagation of AHSV. In this case 200-500 µl (10-200 $TCID_{50}$ /ml) of virus was added to cells suspended in Schneider's media, containing 10% FCS, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and Ampothericin B (4 µg/ml). The infected cells were incubated at 28°C in a 'normal' atmosphere for 7 days. As AHSV does not cause CPE in KC cells, virus propagation was assessed by analysis of a dsRNA genome profile after 1% agarose gel electrophoresis (AGE) (see Section 2.5) or real-time RT-PCR (see Section 2.8).

2.4: Extraction of viral dsRNA

During any manipulation involving RNA, special care was taken to prevent RNase contamination, to minimise RNA degradation. Also, great care was taken to avoid cross-contamination of different strains.

2.4.1: Trizol® regent (Invitrogen) extraction method

This method, previously described by (Attoui *et al.*, 2000), was used to extract dsRNA of AHSV from infected mammalian cells after 70-100 % CPE was observed, or from insect cells at 7 days post infection. AHSV propagated in either mammalian or insect cells remain mainly cell associated and large quantities of viral dsRNA are therefore expected to be extracted using this method.

Briefly, cells from infected cell cultures were harvested, pelleted by centrifugation at 1000 *g* for 5 min, then vigorously re-suspended in 1 ml of Trizol® reagent (Invitrogen). The solution was transferred to a 1.5 ml eppendorf tube and 200 µl of chloroform was added and mixed by vortexing, followed by incubation on ice for 10 min. After centrifugation at 10,000 *g* for 10 min, the top RNA containing aqueous phase was carefully removed into a fresh eppendorf tube and mixed with 900 µl of isopropanol. The mixture was incubated at -20°C for 2 hours and the precipitated RNA was collected by centrifugation at 10,000 *g* for 10 min, washed with 1 ml of 70% ethanol, air dried and dissolved in 100 µl of nuclease free water (NFW). Single stranded RNA (ssRNA) was removed by overnight precipitation with 2M LiCl (Sigma) at 4°C, followed by centrifugation at 10,000 *g* for 5 min. Approximately 200 µl of the supernatant, containing dsRNA, was recovered into a fresh eppendorf tube and mixed with an equal volume of isopropanol and 50 µl of 7.5M ammonium acetate

solution. dsRNA was allowed to precipitate at -20°C for 2 hours and was then collected by centrifugation at 10,000 *g* for 10 min. The RNA pellet was washed with 70% ethanol, air dried and reconstituted in 50 µl NFW. The resulting dsRNA preparation was either used immediately or stored at – 20°C.

2.4.2: Robotic extraction

Total nucleic acid was extracted from tissue culture supernatant, uninfected horse blood or clarified homogenate of *Culicoides* midges, using a BioRobot Universal (Qiagen). In this method, 240 µl of sample was added to 40 µl of Protease (Qiagen) and 360 µl of Lysis Buffer (Roche). After introduction of the mixture to the BioRobot platform, the 'UNIrcV23A V3.0' protocol was run (as per the manufacturer's instructions). The nucleic acid was eluted in ~50 µl of elution buffer (Qiagen) and used immediately or stored for at -20°C.

2.4.3: RNA extraction from cell monolayers using the RNeasy mini Kit (Qiagen)

The supernatant from infected cell sheets was removed and discarded into appropriate disinfectant. Adherent cells were then lysed with 350 µl RTL Buffer (part of the RNeasy mini Kit, Qiagen), supplemented with β-2-mercaptoethanol (β-ME), collected into 1.5 ml eppendorf tubes and homogenised for 1 min by vortexing. Afterwards, the manufacturer's (Qiagen) "Animal Cells Spin" protocol was followed. Briefly, 350 µl 70% ethanol was added to the cell lysate, mixed, transferred to a spin column (provided) and centrifuged at 15,000 *g* for 30 sec. The RNA, which was now bound to the spin column's membrane, was washed once with 700 µl of RW1 Buffer (provided as part of the Kit) and twice with 500 µl RPE Buffer (provided as part of

the Kit but supplemented with 4 volumes of 96-100% ethanol prior use). The RNA was eluted from the columns with 40 µl of NFW, used immediately or stored at 4°C.

2.5: Agarose gel electrophoresis (AGE)

1% AGE was used for the analysis, and visualisation of nucleic acids separating them in accordance with their respective molecular weights.

All agarose gels were prepared with 1% agarose (w/v) (UltraPure™ Agarose, Invitrogen) in 1x TAE buffer (National Diagnostics) containing 0.5 µg/ml ethidium bromide. Electrophoresis was carried out at 100-130V in 1x TAE buffer at room temperature until the different nucleic acid bands (RNA or DNA) were adequately separated.

2.6: Full amplification of cDNA (FLAC)

2.6.1: Ligation of anchor primer

A 35 bp long synthetic "anchor-primer" oligonucleotide, phosphorylated at its 5' end, (5'p-GACCTCTGAGGATTCTAAAC/iSp9/TCCAGTTTAGAATCC-OH3') was ligated to the 3' terminus of purified viral RNA, as described by (Maan *et al.*, 2007b). The "anchor-primer" contains a central flexible phosphoramidite spacer region (/iSp9/) which spans a section of three nucleotides allowing the two complementary halves of the synthetic molecule to form a hairpin like structure. This allows self-priming of cDNA synthesis of the ligated RNA molecule.

The ligation reaction was carried out in a total volume of 10 µl and consisted of 7 µl dsRNA (500-1000 ng), 1 µl anchor primer (500 ng), 1 µl T4 RNA ligase buffer (10 x)

(New England Bio Labs) and 1 µl T4 RNA ligase (10 U) (New England Bio Labs). The reaction was incubated at 4°C overnight.

2.6.2: Separation and extraction of "anchor-primer" ligated dsRNA segments

The "anchor-primer" ligated dsRNA representing the different AHSV genome segments, were separated by 1% AGE (as described in Section 2.5). The AHSV dsRNA migration pattern during AGE (which is remarkably consistent for all members of the virus species), allows individual separation of Seg-1 and Seg-10 while the remaining segments migrate as closer groups or co-migrate (Figure 3.1). The AHSV genome segments were therefore excised from the gel (under UV light using a fresh scalpel blade) as five groups, as follows: Seg-1; Seg-2+3; Seg-4+5+6; Seg-7+8+9 and Seg-10. The excised AHSV genome segments were recovered from agarose using an RNaid® kit (MP Biomedicals), which uses a silica binding method, according to the manufacturer's protocol, except that the final wash was performed twice with 50 µl of NFW, for maximum recovery. An equal volume of isopropanol, 1/10th volume of 3M sodium acetate and 0.2 µg/µl of glycogen were added for precipitation. The mixture was incubated for 5-10 min at room temperature followed by centrifugation at 10,000 *g* for 10 min, to pellet the dsRNA. Finally, the pellets were washed with 500 µl of chilled ethanol, air dried and re-suspended in 11 µl of NFW. Subsequent manipulations with the dsRNA were performed immediately.

2.6.3: dsRNA heat denaturation

The single strands of dsRNA molecules prepared as described above were separated by heating at 95°C for 5 min followed by rapid cooling on ice to prevent re-naturation. The denaturated dsRNAs were subjected to further applications immediately.

2.6.4: Synthesis of cDNA

Strands of viral RNA (11 µl) were denaturated as described in Section 2.6.3. First-strand cDNA synthesis was initiated from ligated self-priming "anchor-primer" (section 2.6.1) and was carried out using a Reverse Transcription System (Promega) as follows: 9 µl of RT mix, containing 4 µl of 25 mM $MgCl_2$, 2 µl of dNTPs mix (10 mM of each dNTP), 0.5 µl of AMV reverse transcriptase (22 U/µl), 0.5 µl of RNAsin (40 U/µl) and 2 µl of RT buffer (x10). The reaction was carried out in total volume of 20 µl. Reactions were incubated allowing for annealing of the self-priming molecule at 25°C for 10 min, followed by reverse transcription at 37°C for 40 min, than 40°C for 10 min. The cDNA generated was diluted with 50 µl of NFW and used for PCR amplification, or stored at -20°C for further applications.

2.7: PCR amplification

2.7.1: Amplification of full-length genome segments

Full-length genome segments amplification was primed by 5-15-1 primer (5'-GAGGGATCCAGTTTAGAATCCTCAGAGGTC-3') and was carried out using PCR Extender System (5 PRIME). The 5-15-1 primer is complementary to ligated "anchor-primer" molecule facilitating full-length amplification of genome segment.

The reaction was performed in total volume of 20 µl in a reaction mixture that contained:

2 µl	reverse transcribed cDNA product (see section 2.6.4)
2 µl	10x PCR buffer (tuning buffer with Mg2+)
0.4 µl	dNTPs (10 mM of each dNTP)
0.4 µl	5-15-1 primer (100 µM)
0.2 µl	PCR extender polymerase mix (5 U/µl)
15 µl	NFW

The cycling parameters (Eppendorf or Biometra thermocyclers) of the reaction were as follows:

Temperature	**Time**	**Cycles**
94°C	2 min	1 cycle
94°C	15 sec	30 cycles
62°C	30 sec	
68°C	5 min (for Seg-1 and Seg-2+3) or 2 min (for remaining genome segments)	
72°C	10 min	1 cycle
4°C	hold	

To analyse the cDNA products of the PCR reaction, 5 µl of the final reaction were mixed with 1 µl of 6x loading dye and run on agarose gel as described in Section 2.5.

2.7.2: Genome segment 1 amplification

On some occasions the full length cDNA product from Seg-1 was ‘weak’ or undetectable after 1% AGE. In these cases smaller overlapping fragments of the segment were amplified from the cDNA in separate reactions. Four sets of primers with similar melting temperatures were designed to amplify overlapping fragments of Seg-1 (listed in Table 2.3). Primer 5-15-1 was modified to ensure amplification of both termini as well as to maintain the melting temperature. The Seg-1 fragments were amplified using the same reaction mixture and thermal profile (2 min elongation step) as described in Section 2.7.1.

Table 2.3: Genome segment 1 amplification primers.

Primer set no	Primer nomenclature	Sequence 5'→3'	Tm	The total length of amplified product (illustration)
1	MOD-5-15-1 AHSV/S-1/1088-1109R	CCAGTTTAGAATCCTCAGAGGTC CGTGACTTATTTTTGTTGGATC	61.9 59.9	
2	AHSV/S-1/1051-1070F AHSV/S-1/2557-2577R	CCTGAAAATGTTAAAAGCGG GTTTGAACTTGAGATCTCAGG	61 57.9	
3	AHSV/S-1/2137-2157F AHSV/S-1/3172-3192R	GGTTTCRCCTACGGATGGAAG CTTGGTCTCCAACCTTGCAAC	63.6 65.8	
4	AHSV/S-1/3038-3059F MOD-5-15-1	GCCGTRAGAAGTTTGCCTCTGG CCAGTTTAGAATCCTCAGAGGTC	62.7 61.2	

List of primers used for amplification of overlapping fragments of Seg-1.

2.7.3: Amplification of fragments of Seg-2 for sequencing

Fragments of AHSV Seg-2 were amplified using serotype-specific primers as listed by (Maan *et al.*, 2011a) and SuperScript® III One-Step RT-PCR Platinum®*Taq* HiFi kit (Invitrogen) which included a high fidelity polymerase mix with buffer (2x Reaction mix) and $MgSO_4$. Reaction mix for 50 µl total reaction volume:

25 µl	Reaction mix (2x)
7.5 µl	MgSO4 (5mM)
1 µl	SuperScript® III One-Step RT-PCR Platinum ® TaqMix
1 µl	Forward primer (10 µM)
1 µl	Reverse primer (10 µM)
8.5 µl	NFW
6 µl	Previously denaturated dsRNA as described in section 2.6.3 prior to addition to the reaction mix

Cycling parameters (Eppendorf or Biometra thermocyclers):

55°C	30 min	1 cycle
94°C	15 s	1 cycle
94°C	30 s	40 cycles
55°C	30 sec	
68°C	1 min/Kilobase (Kb) product length	
68°C	5 min	1 cycle
4°C	hold	

To analyse the products of the PCR reaction, 5 µl of the reaction were mixed with 1 µl of 6x loading dye and analysed by 1% AGE as described in Section 2.5

2.7.4: Generation of PCR products for cloning of the VP2 open reading frame (ORF)

A set of 8 primers pairs were designed for amplification of the VP2 open reading frame (ORF) (for AHSV 1-3 and AHSV 5-9) (Table 2.4). These included Xma1/Sma1 restriction sites (on the forward and reverse primers respectively) and the Kozak sequence (forward primers).

This part of the primer's sequence was not complementary to the template but was included to facilitate cloning (restriction sites) or translation (Kozak sequence). Extra random nucleotides (not complementary to the template) were added next to the restriction site, to enhance the activity of the restriction enzymes. Start and stop codons mark the beginning of the primers which are complementary to the cDNA template and therefore involved in its amplification.

cDNAs of Seg-2 of the respective reference strains of AHSV (for cDNA synthesis see Section 2.6.4) were used as a template for the amplification reaction, which was carried out using AccuPrime™ *Taq* DNA Polymerase System (Invitrogen).

Reaction mix for 50 µl total reaction volume:

5 µl	AccuPrime™ PCR Buffer I (10X), includes dNTPs and 50 mM $MgCl_2$.
0.2 µl	AccuPrime™ Taq DNA Polymerase
1 µl	Forward primer (10 µM)
1 µl	Reverse primer (10 µM)
42 µl	NFW
1 µl	cDNA template

The cycling parameters (Eppendorf or Biometra thermocyclers) were:

Temperature	Time	Cycles
94°C	2 min	1
94°C	30 s	35
60°C	30 s	
68°C	30 s	
4°C	hold	

Table 2.4: List of primers used for amplification of the VP2 open reading frame.

AHSV serotype	Oligo name	Sequence (5'-3')
Forward primers		
AHSV-1	AHSVvp2(1)f	ATATCCCGGGACCATGGCGTCTGAATTTGG
AHSV-2	AHSVvp2(2)f	ATATCCCGGGACCATGGCGTCTGAATTTGG
AHSV-3	AHSVvp2(3)f	ATATCCCGGGACCATGGCTTCGGAATTCGG
AHSV-5	AHSVvp2(5)f-2	ATATCCCGGGACCATGGCTTCAGAGTTTGG
AHSV-6	AHSVvp2(6)f-2	ATATCCCGGGACCATGGCTTCCGAATTTGG
AHSV-7	AHSVvp2(7)f	ATATCCCGGGACCATGGCTTCTGAGTTTGG
AHSV-8	AHSVvp2(8)f-2	ATATCCCGGGACCATGGCGTCGGAGTTTGG
AHSV-9	AHSVvp2(9)f	ATATCCCGGGACCATGGCGTTCGAGTTTGG
Reverse primers		
AHSV-1	AHSvp2(1)r	ATATCCCGGGTTACTCTATCTTCGACAATAAC
AHSV-2	AHSvp2(2)r	ATATCCCGGGTTACTCTATTTTGGATAGTAG
AHSV-3	AHSVvp2(3)r-2	ATATCCCGGGTTATTCAGTTTTCGCAAGCAG
AHSV-5	AHSvp2(5)r	ATATCCCGGGCTACTTTTCTGTCTTGGCGAG
AHSV-6	AHSVvp2(6)r-2	ATATCCCGGGCTATTCAGCTTTTGCCATCAG
AHSV-7	AHSvp2(7)r	ATATCCCGGGTTACTCTGCCTTGGATAACAG
AHSV-8	AHSVvp2(8)r-2	ATATCCCGGGCTACTTCTCTGTTTTGGCTAGT
AHSV-9	AHSvp2(9)r	ATATCCCGGGCTACTGTTCGATCTTGGC

Extra nucleotides are indicated in **green**, restriction site (Xma1/Sma1) – in red, Kozak sequence – in **purple** and start/stop codons in **blue**.

2.7.5: Colony screening by touch PCR

Half a microliter of bacterial mini-culture (as described in Section 2.9.7.1) was then used as DNA template for a PCR reaction in a total volume of 10 µl. GoTaq® Hot Start Polymerase (Promega) Kit was used for the amplification in the following reaction mix:

2 µl	5X Green GoTaq® Flexi Buffer
0.4 µl	MgCl2 (part of the kit)
0.2	dNTP (part of the kit)
1 µl	Forward primer (10 µM)
1 µl	Reverse primer (10 µM)
0.05 µl	GoTaq® DNA Polymerase
6.65 µl	NFW
0.5 µl	DNA template

The cycling parameters used (Eppendorf or Biometra thermocyclers) were:

Temperature	Time	Cycles
95 °C	2 min	1
95 °C	30 sec	30
55 °C	1 min	
72 °C	1 min	
72 °C	5 min	1
4 °C	hold	

The product of the PCR reaction was analysed by adding 5 µl of the product to 1 µl of 6x loading dye, followed by 1% AGE, as described in Section 2.5.

2.8: TaqMan real- time RT-PCR for detection and identification of AHSV

The real time RT-PCR follows the same general principle as a conventional RT-PCR, with reverse transcriptase and PCR amplification steps (Saiki *et al.*, 1988). However, the PCR is modified so that amplification of the cDNA products can be detected in real time. The chemistry of TaqMan based real-time PCR assays requires a third oligonucleotide, the 'probe', in addition to the forward and reverse primers. The probe is labelled with a fluorescent reporter dye e.g. 6-Carboxyfluorescein (FAM) at the 5'

end and with a quencher at the 3' end e.g. Black Hole Quencher (BHQ). When the reporter and the quencher remain in close proximity (attached to the probe), no fluorescence signal is released upon excitation at the reported dye's emission wavelength, as the energy is transferred to the quencher by fluorescence resonance energy transfer (FRET). During the annealing step of the PCR, the probe anneals to a specific target sequence on the complementary strand of the template, which is situated in between the two primer binding sites. As the polymerase extends the primer on the same strand as the probe, its 5' exonuclease activity cleaves the probe, starting from its 5' end, releasing the reporter dye into solution. When the dye is subsequently excited at its emission wavelength, the energy is no longer absorbed by FRET but is released from the fluorophore as fluorescence (Figure 2.1).

The fluorescence signal can be detected and measured in real time using a real-time thermal cycler. With each cycle the fluorescence intensity increases in line with the increase in amplicon concentration. The measured levels of fluorescence are plotted against the thermal cycle number and a threshold level of fluorescence is established within the linear amplification phase, which represents positive detection of the targeted DNA template. The number of cycles required to cross this threshold level (the "Ct" value) is dependent on the amount of template in the original sample. The greater the amount of the initial template, the lower the Ct value for that sample. Therefore, with appropriate controls and evaluation, real-time RT-PCR assays can be used in a quantitative measure of template copy numbers.

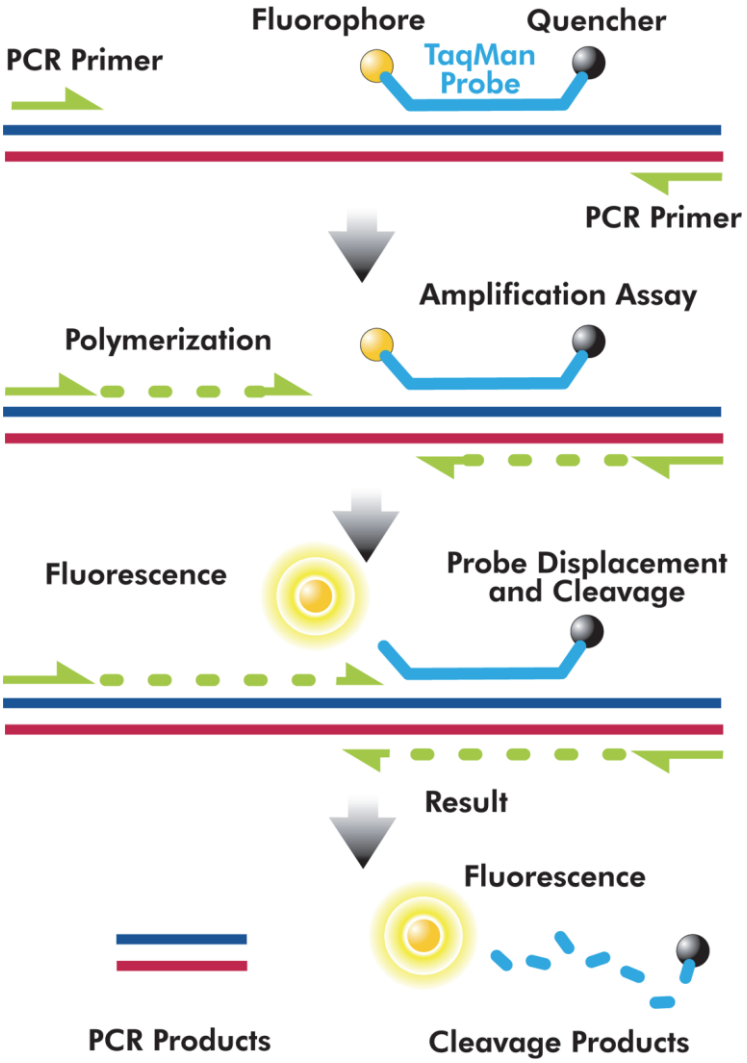


Figure 2.1: The principle of real-time TaqMan PCR chemistry. The illustration was accessed via www.chem.agilent.com

Standard guidance, as described below, was adopted when designing TaqMan assays:

- Amplicons length spanned between 70-250 bp, measured from the 5' end of each primer.
- Primers were designed to be between 15-30 bp in length, with the theoretical melting temperature (Tm) of ~60°C, while probes were designed as 20-30 bp in length with Tm 10°C higher to the primers pair.

- Thymines (T) were not included at 3' ends of the primers, nor guanines (G) at the 5' ends of probes.
- Runs of repeated nucleotides were avoided in both primers and probes, in particular runs of G residues in probes.
- Probes were designed to contain more C than G residues with total of 30-80% of those residues.

All real-time RT-PCR probes designed in for this study were labelled with FAM at the 5' end and with black hole quencher 1 (BHQ-1) at the 3' end. All amplifications were performed with SuperScript III Platinum *Taq* One-Step qRT-PCR kit (Invitrogen) in a total volume of 25 µl (group assays) or 20 µl (type assays) with the addition of $MgSO_4$ as described in detail in Chapter 4.

2.8.1: Preparation of standards

Viral dsRNA (RSArrah/01) was purified as prescribed in section 2.4.1 and assessed for any ssRNA remains in agarose gel electrophoresis (section 2.5). Very small amounts of residual ssRNA was detected which was not regarded as of practical consequence. Concentration of dsRNA was determined with NanoDrop (Thermo Fisher Scientific) and the number of copies calculated with the formula: $Y=(X/a \times 680) \times 6.022 \times 10^{23}$ where: Y = molecules/µl; X = g/µl dsRNA; a = viral genome length in nucleotides; 680 is the average molecular weight per nucleotide of dsRNA.

The dsRNA was diluted in nuclease free water (Promega) to achieve 10^{10} copy number per µl, and further 10-fold dilutions (10^9-10^0 copies per µl) were made in a 1:1000 dilution of RNA extracted from uninfected BHK supernatant, to reduce the loss of the target RNA due to nonspecific binding to plastics. The dilution range of 10^9-10^0 copies per µl were tested with the group specific real-time RT-PCR assays

detecting Seg-1 and Seg-3, to assess their limit of detection, linear regression of Ct values and the efficiency of the group specific assays.

2.9: Cloning of AHSV Seg-2

2.9.1: Production of competent DH5α cells

The DH5-α strain of *Escherichia coli* (*E. coli*), kindly provided by Dr Terry Jackson's group at IAH, were 'streaked' onto a fresh, pre-warmed Luria Bertani (LB) agar plate (obtained from Central Service Unit, IAH Pirbright) and incubated at 37°C overnight. A single bacterial colony was picked with a sterile toothpick into 25 ml of LB broth in a 250 ml conical flask, placed in orbital shaker set at 200 rpm and grown at 37°C for 8 hours. Ten ml of the resulting bacterial culture was diluted into 250 ml of LB both in a 1 litre flask and grown overnight at 18°C, with shaking until an O.D. at 600nm reached 0.55. At this point, the culture was crash cooled in an water-ice bath for 10 minutes and the bacterial cells were collected by centrifugation at 2,500 *g* for 10 min. Care was taken to remove all remains of the culture supernatant before the bacterial cells were washed in 80 ml of ice cold Inoue Transformation Buffer (ITB) (buffer composition in Appendix II) sterilised by passage through a 0.45 µm filter (Nalgene) prior to use. After washing and subsequent centrifugation at 2,500 *g* for 10 min for collection of the cells, the supernatant was carefully removed and cells were re-suspended in 20 ml ice cold, sterile ITB. Then, 1.5 ml Dimethyl sulfoxide (DMSO) was added, gently mixed in by swirling, and the mixture was incubated on ice for 10 min. Aliquots of 200 µl of the competent DH5-α were immediately frozen by flash-freezing in dry-ice/ethanol mix and stored at -80°C until required. All bacterial

manipulations were performed using standard microbial techniques to prevent contamination of the cultivated bacterial strain.

2.9.2: Cloning into pSC11 vaccinia transfer vector

cDNA inserts of the VP2 gene from AHSV-2, 5, 6, 8 and 9 (RSArrah/02, RSArah5/03, RSArah6/03, RSArah8/03 and PAKrah9/03) were prepared as described in Section 2.7.2 and were cloned into pSC11 (kindly provided by Dr Javier Castillo-Olivares) (Chakrabarti *et al.*, 1985) (Figure 2.2).

To facilitate cloning, the plasmid was linearised using XmaI (Promega) (section 2.4.6.3) while the purified inserts were digested using the same restriction enzyme (RE) to create directly equivalent and compatible "sticky ends" on both plasmid and insert. Before ligation of the "sticky ends", the 5' ends of the vector were dephosphorylated using alkaline phosphatise (SAP) (Promega) according to the manufacturer's instructions, to prevent self-ligation of the RE treated vector. Ligations were conducted with T4 DNA ligase(Promega) as described in Section 2.9.5. The recombinant plasmids were then used to transform cells of the DH5-α strain of *E. coli* (Section 2.9.6). The resulting recombinant plasmids were purified (Section 2.9.7.2) and the insert was sequenced (Section 2.10), using vector ($P_{7.5}$F, TKR) and AHSV Seg-2 specific primers. This confirmed the inserts' orientation and integrity by comparisons to Seg-2 of the original virus strain.

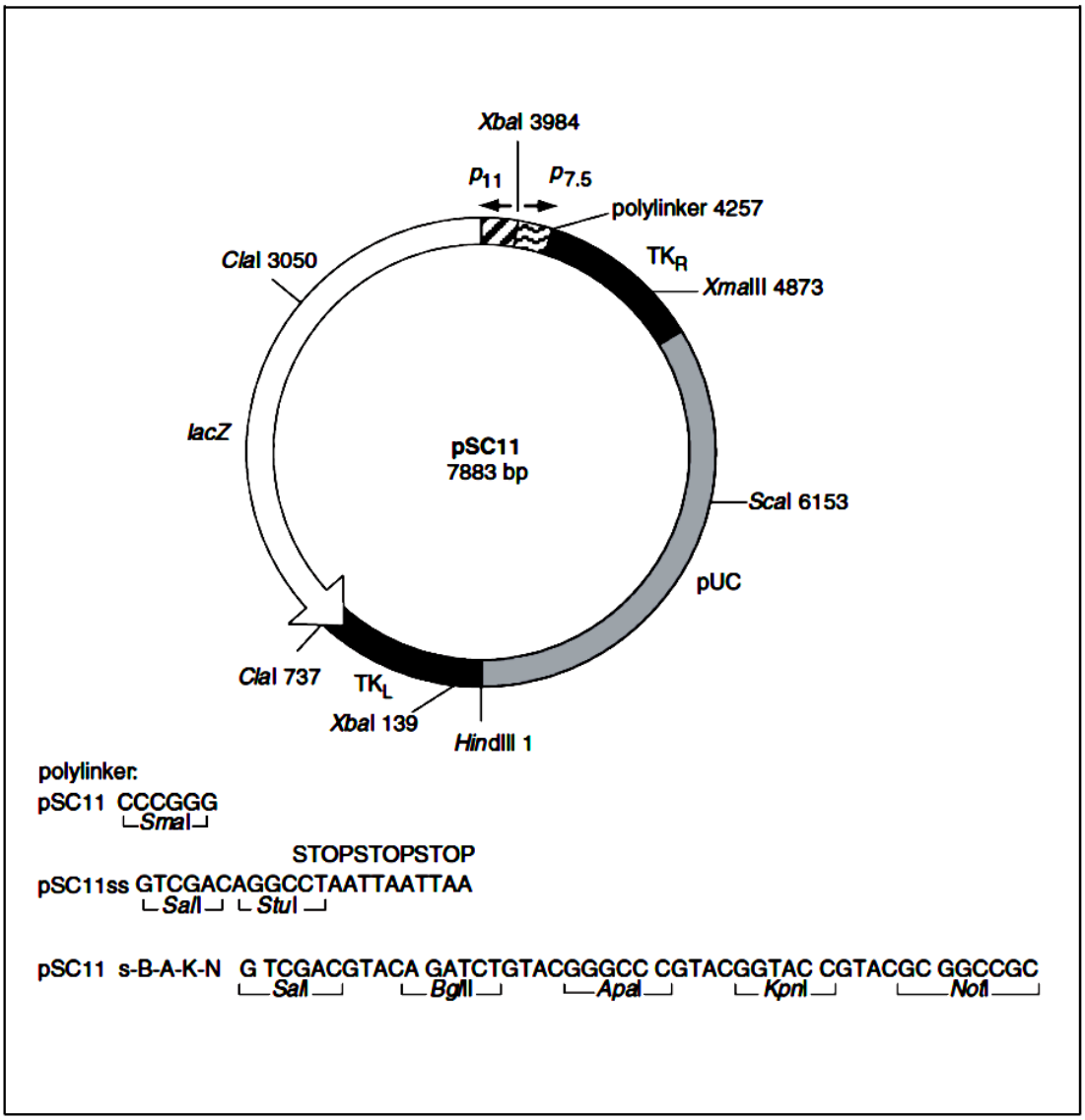


Figure 2.2: A schematic diagram of the plasmid transfer vector pSC11. (Earl *et al.*, 2001).

2.9.3: Restriction endonuclease digestion of DNA

Restriction digestion (RE) reactions were performed according to the manufacturer's instructions (Promega) in a total volume of either 20 or 50 µl. A typical 50 µl reaction included: 0.5 µg of the vector/insert DNA, 0.5 µl of bovine serum albumin (BSA), 5-10 units XmaI, 5 µl 10x Restriction Enzyme Buffer B (Promega) and NFW up to the final volume. The reactions were incubated in a water bath at 37°C for 1.5-2 hours.

The efficiency of the digests and digested products were assessed by 1% AGE as described in Section 2.5.

2.9.4: Gel purification

PCR and RE digest products, as well as SAP treated, linearised vectors, were analysed and separated by 1% AGE (Section 2.5). The DNA fragments were excised from the gel and purified for subsequent applications using GFX™ PCR DNA and Band Purification Kit (GE Health care), according to the manufacturer's instructions. The purified DNA was quantified using NanoDrop®ND-1000 spectrophotometer (Labtech) and used immediately or stored at -20°C till required.

2.9.5: DNA ligation

Both nucleic acid components of a ligation reaction, dephosphorylated plasmid and insert, were digested using the same restriction sites, prior to the reaction. After purification (Section 2.9.4), they were mixed in ratios of vector to insert of 1:1, 3:1 or 1:3, in a reaction volume of 10 µl, containing: 5 µl 2x Rapid Ligation Buffer (Promega), 1-3 units of T4 DNA ligase (Promega) and NFW up to the final volume. Ligations were incubated at room temperature for 1-2 hours, or overnight at 4°C before transformation into competent DH5α strain *E. coli*.

2.9.6: Transformation of Bacteria

A hundred microliters of competent DH5α cells (prepared as described in section 2.9.1), thawed on ice, were transformed with 1-3 µl pre-chilled ligation reaction, incubated on ice for 30 min, heat shocked at 37°C for exactly 45 seconds in a water bath and returned on ice for two more minutes incubation as described by (Sambrook,

1989). One ml of sterile SOC medium (CSU, Pirbright) was added and the tube was incubated on an orbital shaker (200 rpm) for 1 hour at 37°C. The cells were spread (50-200 µl) onto pre-warmed LB agar plates containing ampicilin (100 µg/ml), inverted and incubated overnight at 37°C.

2.9.7: Isolation of plasmids from recombinant bacterial clones

2.9.7.1: Bacterial mini-culture

Bacterial colonies were numbered and picked using a sterile pipette tip, into 100 µl Luria Bertani (LB) medium supplemented with ampicillin (100 µg/ml) dispensed into wells of a 96 well PCR plate and sealed with a PCR film. For propagation of bacteria the plates were incubated on an orbital shaker (200 rpm) at 37°C for 1 hour. The resulting bacterial mini-cultures were screened by colony PCR (Section 2.7.4) for insert detection or used to inoculate a miniprep culture.

2.9.7.2: Bacterial minipreps

Recombinant bacterial clones that were identified by touch PCR were cultured in 12 ml of LB broth (containing 100 µg/ml ampicillin), with shaking at 200 rpm, overnight at 37°C. The bacterial cells were harvested by centrifugation (3,000 *g*, 15 min) and recombinant plasmids were isolated from the pellets using silica columns from a QIAprep® Spin Miniprep kit (Qiagen) in accordance to the manufacturer's instructions. The purified plasmids were eluted in 50 µl NFW and were quantified using a NanoDrop®ND-1000 specrophotometer (Labtech) then stored at -20°C till required. The nucleotide sequence of the cloned inserts was confirmed by sequencing.

2.10: Nucleotide sequencing

2.10.1: Sequencing of PCR products

All sequencing reactions were set up in 96 well plates using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) with a 10 µl total reactions volume as follows:

0.25 µl	BigDye Terminator Sequencing Mix v3.1 (2x)
1.88 µl	BigDye Terminator Sequencing Buffer v3.1 (5x)
1 µl	Primer (10 µM)
20-150 ng/µl	PCR product/plasmid

The products were amplified according to following thermal programme:

96°C	1 min
96°C	20 sec
50°C	5 sec
60°C	4 min
4°C	hold

Sequencing primers, as listed in Appendix III, were designed based on the published sequences for the AHSV genome segments (Table 2.5) and were generally 18-25 nucleotides in length and 40-60% GC content. All primers were supplied by Sigma.

After the sequencing PCR reactions had completed, the products were ethanol precipitated (Section 2.10.3) and re-suspended in 20 µl Hi-Di Formamide. The cDNA sequences were generated using an automated ABI 3730 DNA sequencer (Applied Biosystems) and analysed (as described in Section 2.11).

Table 2.5: Accession numbers of sequences used for designing primers.

	AHSV-1	AHSV-2	AHSV-3	AHSV-4	AHSV-5	AHSV-6	AHSV-7	AHSV-8	AHSV-9
Seg-1	NC006021 FJ011107 FJ196584	FJ196584							U94887
Seg-2	AY163329 FJ011108 AM883165	EU303127 AY163332 FJ196585	DQ868772 U01832 Z26316 EU303128	EU046574 D26570 DQ868773 M94680	AY163331	AF021235 NC005996 EU303130 DQ868774	AY163330 AY159940 AY159942 AY159946 DQ118704	DQ868775 EU303131 AY163333	DQ868776 AF043926
Seg-3	AM883166 EU303138	EU303139	EU303136 EU303133	D26572 EU303137		AF021236 EU303142		EU303143	
Seg-4	AM883167 FJ011110	EU303151	EU303152 EU303147	D14402 AF246225		EU303154	EU303155	EU303156	
Seg-5	FJ183368 FJ011111	EU303164	EU303161 EU303158	D11390 EU303162		U73658 NC006020		EU303168	U01069
Seg-6	FJ011112 EU303175 AM883169	EU303176	EU046575 EU046576 DQ868777	EU046578 DQ868778 D26571 M94731		EU046577 DQ868779 AF021237	EU303179	DQ868780 EU303180	DQ868781 U74489
Seg-7	AM883170 FJ011113		U19881						NC006019
Seg-8	AM883171 FJ011114		AF545433	X56676 NC006011		AF021238	D12533		S69829 U90337
Seg-9	FJ183372 FJ011115		AF545434						M69090
Seg-10	FJ011116 AF276702 AF276695	EU433543 EU433521 EU433409	DQ868782 EU433408 EU433435	EU046579 EU433419 AF276696	EU433406 EU433421 EU433459	DQ868784 EU433523 EU433460	U60190 EU433468 EU433510	DQ868785 EU433517 EU433461	DQ868786 EU433470 EU433528

2.10.3: Sequencing reaction cleanup

The sequencing PCR products were 'cleaned up' using ethanol precipitation. Briefly, 5 µl 125 mM EDTA and 30 µl chilled 100% ethanol were added to each well of the 96-well plate containing the PCR products. The contents were mixed and incubated in the dark at room temperature for 15 min. The samples were centrifuged (3,000 *g*, 30 min) and the pellets were washed with 30 µl of chilled 70 % ethanol (centrifugation 1650 *g*, 15 min). After removal of the supernatant, the pellets were vacuum dried, dissolved in 20 µl Hi-Di formamide and subjected to sequencing or stored, until needed at -20°C.

2.11: Sequence data analysis

In order to generate the full length consensus sequence, sequence data obtained from the ABI sequencer was assembled using SeqManII sequence analysis software (DNAstar v.5). All sequences were appropriately annotated with the virus name, serotype and genome segment number. Nucleotide and amino acid sequences were analysed using BioEdit (Hall, 1999) while multiple sequence alignments were performed using ClusterW (Thompson *et al.*, 1994), MUSCLE 3.7 (available online at http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=muscle) and VectorNTI (Invitrogen) software packages. MEGA 5 software (Tamura *et al.*, 2011), was used for selection of best fitted nucleotide and amino acid substitution models for each of the genome segments respectively. The same software package was also used to infer maximum-likelihood phylogenectic trees, based on the best-fit model.

Neighbour-joining phylogeny and nucleotide or amino acid sequence distances between different strains, were also estimated using MEGA 5 software. Estimations of selective pressure on the protein coding sequences were based on calculations of the global ratio of

non-synonymous (d_N) to synonymous (d_S) substitutions per synonymous site (the d_N/d_S ratio), using a single-likelihood ancestor-counting (SLAC) method (Kosakovsky Pond & Frost, 2005). This programme was accessed through the on-line Datamonkey server (http://www.datamonkey.org) (Delport *et al.*, 2010; Pond & Frost, 2005). The same approach was used to infer nucleotide substitution rates (expressed as nucleotide substitutions per site) of protein coding sequences from individual genome segments. BioEdit software (Hall, 1999) was used to generate hydrophobicity profiles for the individual viral protein sequences, based on a Kyte and Doolittle hydrophobicity scale, where values above zero indicate a hydrophobic character (Kyte & Doolittle, 1982).

2.12: Generation of recombinant MVA

Foreign genes can be introduced into modified vaccinia Ankara virus (MVA) by homologous recombination within the thymidine kinase (TK) gene, when CEF cells, previously infected with MVA, are transfected with pSC11 vector carrying the foreign gene (Chakrabarti *et al.*, 1985). This mechanism was used to produce recombinant MVA expressing VP2 of AHSV-9 (MVA/AHSV-9/VP2) by infection of susceptible cell with wild type MVA followed by transfection with pSC11 (pSC11/AHSV-9/VP2) carrying a VP2 gene insert from AHSV-9 (PAKrrah/09) (Section 2.12.1). After elimination of wild type MVA (Section 2.12.2) a stock of MVA/AHSV-9/VP2 was prepared (Section 2.12.3) and characterised (as described in Sections 2.12.4 and 2.12.5) before the virus was inoculated into animals (Section 2.13).

2.12.1: Transfection of pSC11/AHSV-9/VP2 in MVA infected cells

Confluent monolayers of chicken embryo fibroblasts primary cell line (CEF), propagated in a 6-well plate were infected with wild type MVA (provided by Dr Javier Castillo-Olivares, PIR, Pirbright) at a multiplicity of infection (MOI) of 0.1. One hour after infection, the cells were washed with serum free (SF) DMEM (without antibiotics) and transfected with 4 µg per well of recombinant pSC11 plasmid (pSC11/AHSV-9/VP2) containing VP2 of AHSV-9 (PAKrrah/09), in 500 µl SF DMEM (without antibiotics) supplemented using 10 µl Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The infection was allowed to progress for two days then the cells were recovered from the wells, pelleted by centrifugation at 1500 *g* for 10 min and re-suspended in 1ml SF DMEM. Virus particles were released from the cells in three freeze-thaw cycles.

2.12.2: Plaque purification

Recombinant MVA/AHSV-9/VP2 virus was purified away from wild type MVA by 4 rounds of plaque assays using the *lacZ* marker and TK negative selection. Selection of recombinant MVA/AHSV-9/VP2 plaques was repeated until no white plaques (wild type MVA) were visible after X-gal staining. The purified plaques were used to generate a "mini-stock" of recombinant MVA/AHSV-9/VP2 virus.

2.12.2.1: LacZ selection

One millilitre of primary chicken embryo fibroblasts (CEF) cells were seeded ($0.45x10^4$/ml) into a 12-well plate one day prior to infection with MVA/AHSV-9/VP2 virus diluted in 0.5 ml DMEM (supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin and 10% FCS). After incubation for 1 h at 37°C the inoculum was removed

and cells were overlaid with 10% DMEM (with antibiotics) containing 1 % w/v low melting point agarose (Invitrogen). The cells were incubated at 37°C with 5% CO_2 atmosphere until plaques became visible, usually after 2 days. Then, a second agarose overlay was added, which in addition to above, contained 400 µg/ml of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) (Invitrogen). After overnight incubation at 37°C with 5% CO_2 atmosphere, the cell sheets were examined for the presence of blue plaques- indicating recombinant MVA, and isolated blue plaques were picked into 1 ml 10 % DMEM.

2.12.2.2: TK negative selection

One round of 'TK negative selection' was carried out on each of the plaque cloned virus strains that were derived from LacZ selection (see above). The viruses were plaque cloned as described for LacZ selection, but using BHK TK^{-} cells with 25 µg 5-bromo-2'-deoxyuridine (BrdU)/ml in both of the agarose overlays to aid the elimination of wild type plaques.

2.12.3: Generation of recombinant MVA/AHSV-9/VP2 virus stock

A primary virus stock of MVA/AHSV-9/VP2 was prepared by infecting CEF cells monolayers in a 6-well plate. After MVA/AHSV-9/VP2 infected cells showed >70% CPE, the cells were scraped from the wells and the cell debris suspension was freeze-thawed three times before needle-homogenistion. The released virus was then titrated (Section 2.12.4) and used as "seed" virus for the generation of a virus stock.

A recombinant MVA/AHSV-9/VP2 virus stock was generated in 175 cm^2 80% confluent monolayers of DF-1 cells. After removal of the medium, cell sheets were inoculated with

100 µl of the virus mini-stock (in 5 ml 10 % DMEM supplemented with antibiotics) and incubated for 1 hour at 37°C. The inoculum was removed and replaced with 40 ml 10 % DMEM with antibiotics. The flasks were incubated until 80-100% CPE was observed, usually 3-4 days. The cells were then scraped from the flasks, collected by centrifugation (3,000 *g* for 10 min), re-suspended in 1 ml 10 % DMEM with antibiotics and freeze-thawed three times to release the virus from infected cells. The titre of the resulting virus stock was determined by plaque assays (Section 2.12.4)

2.12.4: Titration of recombinant MVA

A ten-fold serial dilution of MVA/AHSV-9/VP2 virus, from 10^{-1} to 10^{-8} was made in serum free (SF) DMEM, then used to infect (1 ml/well) confluent CEF cells that had been seeded into 12-well plates ($0.45x10^{4}$/ml) one day prior to infection. The virus, at different dilutions, was allowed to absorb for 1 hour at 37°C with 5 % CO_2. The inoculum was then removed and the cells were overlaid with 10% DMEM (with antibiotics) containing 1% w/v low melting agarose (Invitrogen). The infected cells were incubated at 37°C, 5 % CO_2 under the DMEM-agarose layer for 2 days and when plaques started to develop, second DMEM-agarose containing 400 µg/ml of X-gal (Invitrogene) was added. Blue plaques were counted and the virus titre was determined as: Titre (pfu/ml) = Number of plaques / well x Dilution factor.

The final titre of MVA/AHSV-9/VP2 virus was determined using the β-Gal Staining Kit (Invitrogen) according to manufacturer instructions. Briefly, confluent monolayers of CEF cells were infected with serial dilution of MVA/AHSV-9/VP2 virus and overlaid with 10% DMEM (with antibiotics) containing 1% w/v low melting point agarose (Invitrogen) as described above. After 24h incubation the agarose overlay was removed, infected cells were fixed with 1x fixative solution (part of the β-Gal Staining Kit,

Invitrogen) and stained with the mix of staining solution also provided with the kit. The cells sheets were examined until blue plaques were observed and counted. The virus titre (pfu/ml) was calculated as described above.

2.12.5: Functionality of AHSV-9 VP2 expression cassette in MVA/AHSV-9/VP2.

Due to the lack of AHSV-9 VP2 specific antibodies we could not detect directly the expression of AHSV-9 VP2 from MVA/AHSV-9/VP2 infected cells. Instead RT-PCR was used to confirm the presence of AHSV-9 VP2 transcripts in MVA/AHSV-9/VP2 infected cells.

Thus, confluent monolayer of CEF cells were infected with the MVA/AHSV-9/VP2 virus at a MOI ~1 and incubated at 37°C, 5 % CO_2 for 24 hours. mRNA from the infected cells was extracted using RNeasy mini kit (Quaigen) as described in Section 2.4.3. An RT-PCR reaction was set up using SuprScript® III One-Step RT-PCR Platinum ® *Taq* HiFi kit (Invitrogen) as prescribed in Section 2.7.2 using 9A2 primers pair specific to AHSV-9 Seg-2 (Maan *et al.*, 2011a) (10 µM) and 6 µl of extracted mRNA. Standard PCR reactions were performed in parallel on the mRNA transcripts to verify that any amplicons in the RT-PCR were due to the presence of VP2 transcripts and not AHSV-9 VP2 DNA. This PCR reaction was performed with KOD Hot start mastermix' (Novagen) in 25 µl total volume using 12.5 µl of 2x KOD mastermix, 1 µl of the same specific primers as described above and 6 µl of mRNA template. The reactions were incubated in a thermal cycler (Biometra) according to the profile described in Section 2.7.1. The cDNA products were analysed by AGE (Section 2.5).

Expression of functional VP2 from the rMVA was confirmed by detection of virus neutralising antibodies against AHSV-9 VP2 in the serum of MVA/AHSV-9/VP2 immunised animals (sections 2.13 and 2.14).

2.13: Pony immunisation

Two Welsh Mountain ponies were given two 1 ml doses of 3 x 10^8 pfu of MVA/AHSV-9/VP2 per animal, at a 4 week interval (Figure 7.2). On each occasion, 0.5 ml of the dose was injected intramuscularly and 0.5 ml was delivered intradermaly at two injection sites using a needle-less injection device (Injex). Blood samples were collected on week 0 and 6, two weeks after the last immunisation, and the antibody response to VP2 was analysed in serum samples collected from this bleed.

Animal work was performed under a Home Office License at the Animal Health Trust (AHT), Newmarket and was approved by the AHT's Ethical Review Committee.

2.14: Analysis of AHSV9/VP2-specific antibody responses by AHSV virus neutralisation test

Serum samples collected from MVA/AHSV-9/VP2 vaccinated ponies on day 0 and 45 of the experiment (see section above) were heat inactivated for 30 min at 56°C and diluted 1 in 2 (primary screening) or serially diluted in SF DMEM and 50 µl added to 4 replicate wells of a 96 well microtitre plate. The antisera was incubated with 50µl a virus suspension of 100 $TCID_{50}$ of AHSV at 37°C, 5 % CO_2 for 2 hours. This was done with each of the nine AHSV monotypic reference strains (Table 5.1). After the incubation period, 100µl of a suspension of 2 x 10^4 Vero cells in 10% DMEM were added to every

well and the plates were further incubated for 4 days at 37°C, 5 % CO_2. The cell sheets were examined for CPE and the highest dilution of the sera that prevented 50% AHSV infection of the cells was taken as the end-point of the assay. Final serum titres were calculated using the Spearman-Karber method (Finney, 1964).

Chapter 3: Sequencing and phylogenetic analysis of full genome of the nine AHSV reference strains

3.1: Introduction

Since the development of DNA sequencing techniques in Sanger and Gilbert's labs (Maxam & Gilbert, 1977; Sanger & Coulson, 1975; Sanger *et al.*, 1977), the method has undergone a revolution and is becoming more advanced and increasingly accessible as a tool for researchers and diagnosticians. Not only individual genes but also whole genome sequence data are progressively used for detection, characterisation, classification and epidemiological investigation of many pathogens, including particularly viruses (Cook & Holmes, 2006; Cottam *et al.*, 2006; Ikegami, 2012; Nelson & Holmes, 2007).

As the sequence data-set of the *Orbivirus* species (to which AHSV belongs) has expanded, it has become a tool for many research and diagnostic applications. Genome sequence analysis, including phylogenetic comparisons has been used to characterise and classify a number of viruses within the *Orbivirus* species, such as Peruvian horse sickness virus (PHSV), Stretch Lagoon orbivirus (SLOV) and Pata virus (PATAV), as well as novel serotypes of BTV (Attoui *et al.*, 2009b; Belaganahalli *et al.*, 2012; Belaganahalli *et al.*, 2011; Cowled *et al.*, 2009; Hofmann *et al.*, 2008; Maan *et al.*, 2011b). The extensive sequence data set that is now available for BTV, has been used for phylogenetic comparisons of new isolates, to determine the origins of new disease outbreaks and for characterisation of virus reassortants (progeny virus strains that may have novel biological characteristics, generated by genome-segment exchange between different segmented viruses of the same species infecting a single cell) (Allison *et al.*, 2012; Batten

et al., 2008b; De Clercq *et al.*, 2009; Maan *et al.*, 2008; Maan *et al.*, 2007a; Maan *et al.*, 2010b; Mertens *et al.*, 1987; O'Hara *et al.*, 1998; Potgieter *et al.*, 2009).

Sequence data for multiple virus isolates have provided a basis for the development of fast and accurate molecular tests to detect and characterise viral RNA in tissue or blood samples from infected animals. Some of these assays have already been developed for different orbiviruses, including BTV, AHSV and EHDV (Anthony *et al.*, 2007; Maan *et al.*, 2011a; Mertens *et al.*, 2007; Orrù *et al.*, 2006; Quan *et al.*, 2010; Shaw *et al.*, 2007; Zientara *et al.*, 1995a). A thorough knowledge of virus-gene sequences can be applied in investigations of gene / protein expression and their functional roles using recently developed reverse genetic methods for BTV and AHSV (Boyce *et al.*, 2008; Celma & Roy, 2009, 2011; Matsuo *et al.*, 2010; Ratinier *et al.*, 2011; Shaw *et al.*, 2012; van Gennip *et al.*, 2010; Zhang *et al.*, 2010).

Full length, whole genome sequence data is now available for multiple strains of many BTV and to lesser extent EHDV serotypes (Anthony *et al.*, 2009a, b; Anthony *et al.*, 2009c; Maan *et al.*, 2008; Maan *et al.*, 2010b). Although, sequence data have been already obtained for some AHSV strains, certain genome segments, particularly Seg-2 and Seg-10, are better represented in the databases than others (Table 2.5) (Potgieter *et al.*, 2003; Quan *et al.*, 2008; van Niekerk *et al.*, 2001b). At the start of this project, no full length whole genome sequence of an individual AHSV isolate was available.

Multiple BTV serotypes, belonging to different topotypes, recently emerged in Europe (De Clercq *et al.*, 2009; Maan *et al.*, 2008; Maan *et al.*, 2010b; van Rijn *et al.*, 2012; Wilson & Mellor, 2009). Multiple AHSV types have also been identified in East Africa (Chapter 6) (Aklilu *et al.*, 2012; Kazeem *et al.*, 2008; Wilson *et al.*, 2009a), increasing our awareness of the potential risks of an AHSV introduction into naïve equid

populations in Europe. Whole-genome sequence data for a wider spectrum of AHSV isolates would provide a better understanding of sequence variability, distribution, movement and reassortment of different AHSV serotypes / lineages in the field (Chapter 6). An initial dataset that included all of the reference strains for different AHSV serotypes would provide a basis for design of more advanced, high throughput diagnostic methods to detect and characterise (type) novel virus isolates (Chapter 4).

Evolutionary selection pressure can be assessed for a specific open reading frames (ORF) / protein from the ratio of non-synonymous (amino acid changing) to synonymous (silent) changes (d_N/d_S). Under 'diversifying' or 'positive' selective pressure ($d_N/d_S>1$) an organism gains fitness advantage from a higher fixation rate of amino acid changes. In contrast, 'purifying' or 'negative' selection ($d_N/d_S<1$) points towards functional or structural constraints on the protein and a gain by the organism from the preservation of the existing amino acid sequence of the protein. If the ratio of $d_N/d_S = 1$, it corresponds to neutral evolution (no pressure on the protein coding sequence) (Pybus & Shapiro, 2009).

This Chapter describes full length nucleotide and amino acid sequences, generated for the ten genome segments of nine 'historical' reference strains of AHSV (O'Hara, 1994). Phylogenetic analyses of their nucleotide and amino acid sequences, have determined the identity levels for each segment / protein, and revealed the grouping of the different reference strains. The deduced substitution rates and selective pressures (through assessing the d_N/d_S ratio) provide insights concerning the evolutionary constraints on each of the AHSV genes.

3.2: Results

3.2.1: Electrophoretic analysis of the AHSV genome segments

The ten dsRNA genome segments of AHSV were extracted from infected cells and analysed by agarose gel electrophoresis (AGE). The individual AHSV genome segments migrate in this system, at a rate that is primarily dependent on their length / molecular weight. The lengths of the homologous genome segments are also identical, or very similar, for all nine of the AHSV reference strains, and consequently their migration pattern (electropherotype) during AGE is not only highly conserved but is also characteristic of the virus species (Figure 3.1) (Bremer *et al.*, 1990; Mertens *et al.*, 1989a; Mertens *et al.*, 2005; O'Hara *et al.*, 1998). The AHSV genome segments are numbered according to their molecular weight / migration rate during AGE, with Seg-1 being the largest and Seg-10 the smallest. However, genome segments 7, 8 and 9 are relatively similar in size, and cannot be easily separated by AGE (co-migrate).

3.2.2: Full genome sequencing of the nine AHSV reference strains

The full genomes of the nine AHSV reference strains were transcribed and amplified for sequencing purposes using an anchor-primer method (Maan *et al.*, 2007b) as described in Chapter 2 section 2.6. In some of the AHSV strains (RSArrah/03, RSArrah/05, Kenrrah/07, RSArrah/08 and PAKrrah/09) Seg-1 failed to amplify to the level required for sequencing using primers complementary to the ligated “anchor” molecule. To overcome this obstacle, the full length sequence of Seg-1 was reconstructed from four overlapping fragments, as described in Chapter 2 section 2.7.2. AHSV sequence data available from the public domain (NCBI) (Table 2.5) was used to design segment-specific primers for Seg-1, 3, 4, 5, 7, 8 and 9 as listed in Appendix III.

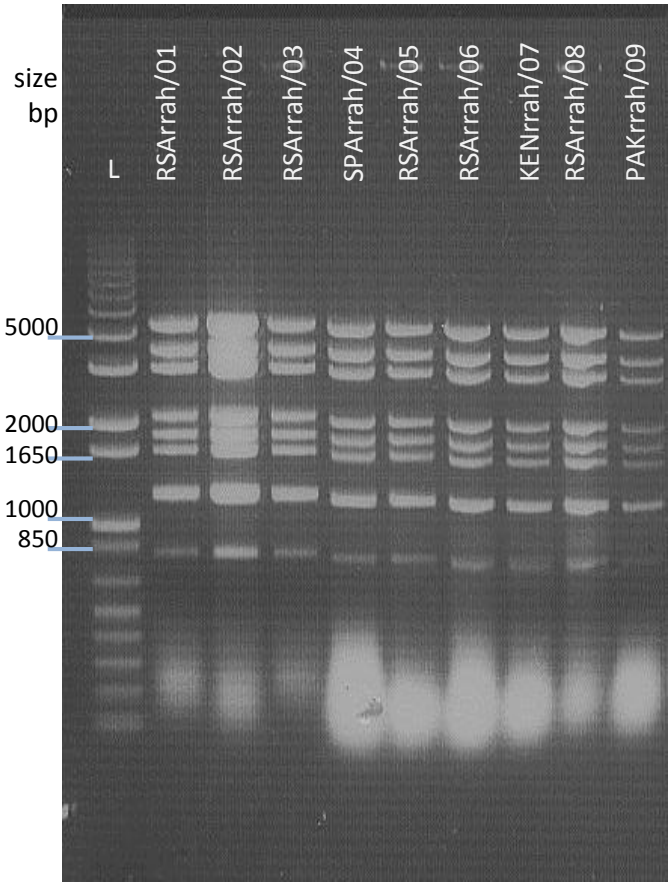


Figure 3.1: The migration pattern of dsRNA genome segments of the nine reference strains of AHSV in 1% agarose gel.
Lane L shows DNA markers – 'TrackIt 1kb Plus DNA Ladder' (Invitrogen). The different reference strains are indicated at the top of the gel. In most isolates ssRNA did not precipitate efficiently during dsRNA extraction (Chapter 2, sction 2.4.1) and is clearly visible as smeared bands at the bottom of the gel with different intensity in the different preparations of RNA from individual isolates. Horizontal bands visible on the photograph are due to poor quality if the image capture system used; these were not visible on the original gel. The gel was run as described in Chapter 2, section 2.5.

Full length sequences were obtained for Seg-2 and 6 of most AHSV serotypes, using sets of primers based on publicly available sequence data, which are designed specifically for each type (Appendix III, Table 2.5). However, for some serotypes (AHSV-2 and 7) only partial sequences of Seg-6 were available. "Primer walking" method was therefore used to obtain the whole sequence of Seg-6. As no sequence data at all was available for Seg-

6 of AHSV-5 (Table 2.5), a universal Seg-6 specific primer (AHSV1-9/S-6/1-22F) was used for initial sequencing reactions. This provided sufficient data for the subsequent design of primers for sequence 'walking', making it possible to obtain full length data for this genome segment. All sequencing primers used in this study, to generate full length data for Seg-2 and Seg-6, are listed in Appendix III. AHSV Seg-10 has previously been shown to exist in three forms/clades: α, β and γ (Martin *et al.*, 1998; Quan *et al.*, 2008; Sailleau *et al.*, 1997b; van Niekerk *et al.*, 2001b). Primers targeting each clade were designed (as listed in Appendix III) and used to determine the full-length Seg-10 sequences.

3.2.3: Sequence analysis

Full length sequences of the entire genomes of nine AHSV reference strains have been generated for use in this study. All sequences discussed refer to positive strands of the viral genome in 5'-3' orientation and are presented as complementary DNA sequences. Analyses of sequence data for each of the respective genome segments is presented in the form of phylogenetic trees and distance matrices, with the overall analyses regarding individual genome segments summarised in relevant tables. Genetic relatedness of the sequences is referred as sequence identity throughout the Chapter.

3.2.3.1: Genome segment 1

AHSV Seg-1 is 3965 nucleotides long in all nine reference strains. The G + C nucleotide content is conserved across all of the strains analysed and varies from 40.5% to 41.4%. The 3' and 5' terminal hexanucleotides of Seg-1 are fully conserved as 5'-GTTTAT…...ACTTAC-3' (Table 3.1), and although the 5' non-coding region (NCR) is also fully conserved as there are two variable nucleotides in the 3' NCR as highlighted in

Table 3.1. The same position start and stop 'TGA' codons are used in each of the Seg-1 sequences analysed (Table 3.1). The Seg-1 ORF is also conserved at the nucleotide level with the lowest percentage identity calculated as 89.1% for RSArrah/05 - PAKrrah/09 and RSArrah/08 - PAKrrah/09, and the highest identity of 99.6% calculated for RSArrah/01 – SPArrah/04 (Table 3.2). The nucleotide sequence variations (single nucleotide polymorphisms - SNPS) are evenly distributed along the alignments, for the strains investigated with no apparent regions of low or high variability (Figure 3.2).

The ORF encoded by Seg-1 spans between nt 14 to 3931 and encodes the VP1 protein (O'Hara et al., 1998) composed of 1305 amino acids with a predicted molecular mass of ~150 KDa (Table 3.1). The protein is mainly hydrophilic but clear hydrophobic regions are distributed along its length (Figure 3.3).

The VP1 is also highly conserved between the nine reference strains, throughout its length (Figure 3.2) with a minimum percentage identity among isolates investigated of 97.6% calculated for RSArrah/06 – PAKrrah/09. Some of the reference strains (RSArrah/01 – RSArrah/02 and RSArrah/01 – SPArrah/04) share almost identical versions of VP1 with 99.9% identity. Such high level of protein identity, especially in comparison to much lower minima for the nucleotide sequences of 89.1%, indicates a high level of functional conservation and the evolutionary importance of the viral polymerase. To support this observation, the rate of non-synonymous (d_N) to synonymous (d_S) substitutions per site were estimated for the protein coding sequence of Seg-1. This calculation can be used to identify signatures of purifying ($d_N/d_S < 1$) or positive ($d_N/d_S > 1$) selection. The results of these calculations indicate strong negative selection on the sequence encoding VP1 ($d_N/d_S = 0.029$) with nucleotide substitution per site (substitution rate) of 8.16×10^{2} (Table 3.22).

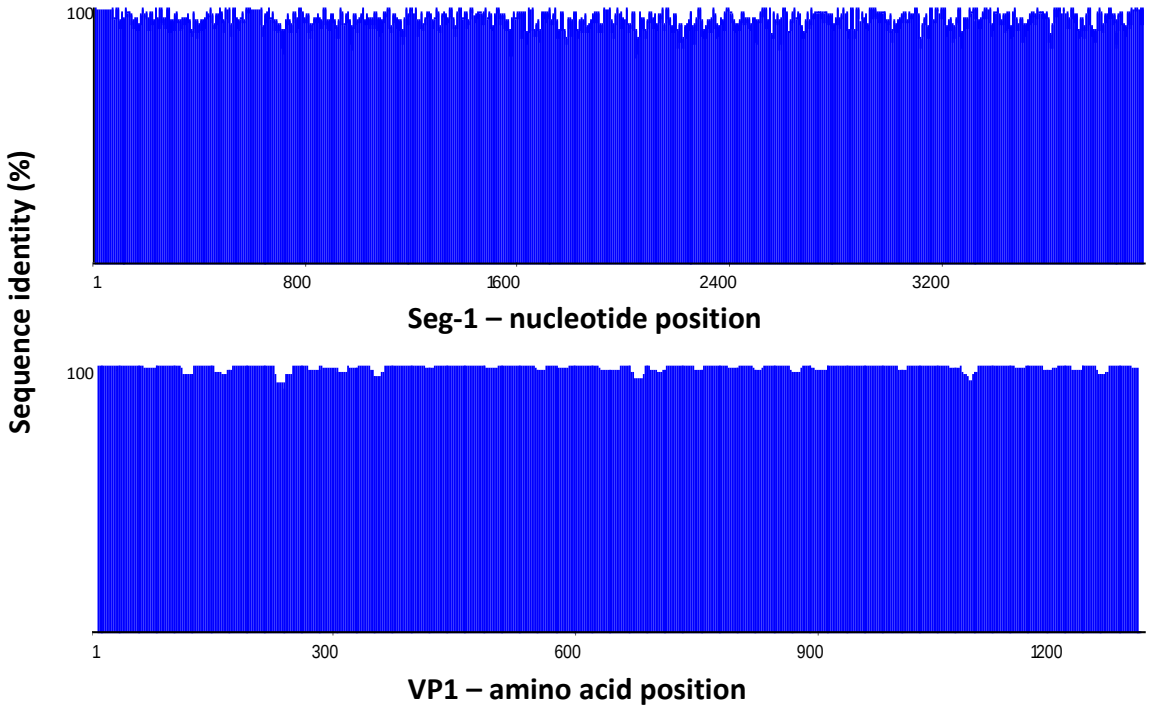


Figure 3.2: Graphical representation of nucleotide and amino acid sequences identities of Seg-1 / VP1.
Low level of nucleotide variations between the strains analysed was detected throughout the length of Seg-2 while VP1 was highly conserved. Alignments and plots were constructed using VectorNTI software (Invitrogen); analytical window size 10.

The high level of nt and aa sequence conservation in Seg-2 between all of the AHSV isolates investigated is reflected in the topology of a Maximum likelihood (ML) phylogenetic tree (Felsenstein, 1981) (Figures 3.5 and 3.5). A neighbour-joining (NJ) analysis (Saitou & Nei, 1987) produced similar results (data not shown). Despite isolates RSArrah/05, RSArrah/06 and RSArrah/08 forming a separate cluster, both nucleotide and amino acid analyses of Seg-1 show very high level of identities, with no evidence of topotype segregation within the limited group of strains analysed (Table 3.2).

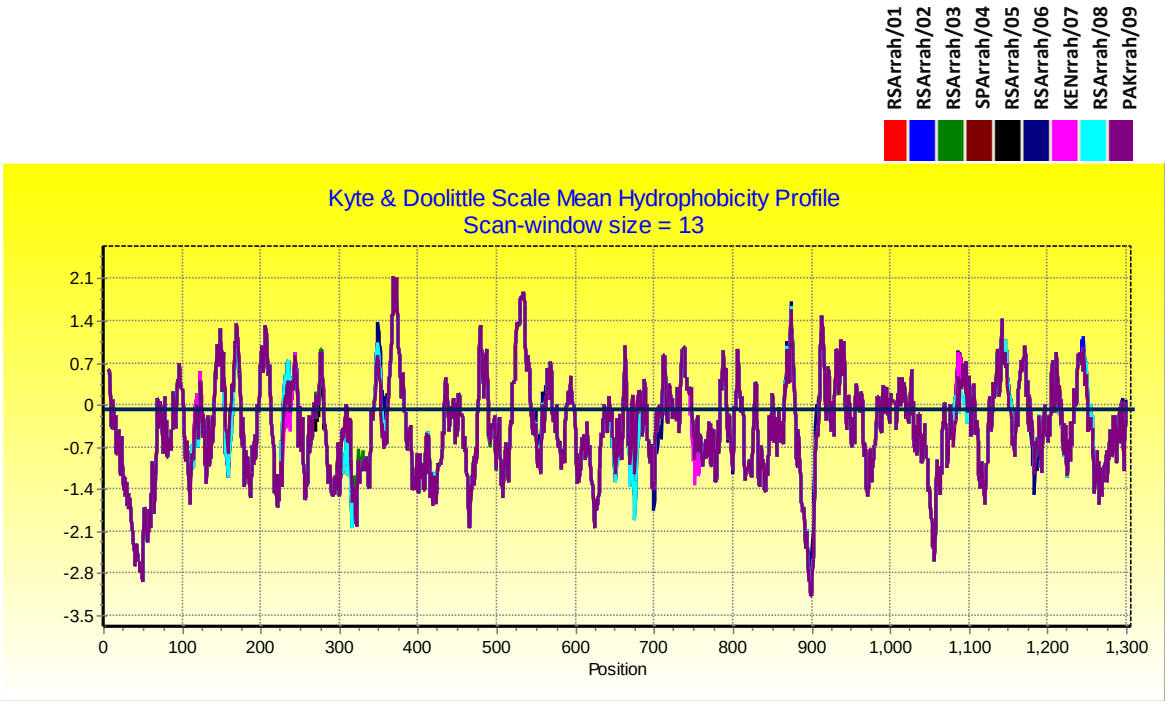


Figure 3.3: Hydrophobicity plot of the AHSV VP1 proteins.
Hydrophobicity plots were inferred for VP1 of each of the AHSV reference strains (represented by different coloured lines as per key) using Kyte and Doolittle Hydrophobicity Scale (Kyte & Doolittle, 1982) accessed through BioEdit (Hall, 1999). As the plots are similar for all of the strains analysed, the lines mostly overlay. Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0.

Table 3.1: Genetic information for Seg-1/VP1 of nine reference strains of AHSV.

AHSV isolate name	Segment length (bp)	Open reading frame (bp)	G+C content (%)	Protein size (aa)	Predicted protein mol mass (kDa)	Stop codon	5' NCR	3' NCR
RSArrah/01	3965	14-3931	41.13	1305	150.1	TGA	**GTTTAT**TTGAGCG	TTAGAACTAGCACCC**CA**CAGCTCAAAAC**ACTTAC**
RSArrah/02	3965	14-3931	41.13	1305	150.2	TGA	**GTTTAT**TTGAGCG	TTAGAACTAGCACCC**CA**CAGCTCAAAAC**ACTTAC**
RSArrah/03	3965	14-3931	40.96	1305	150.2	TGA	**GTTTAT**TTGAGCG	TTAGAACTAGCACCC**TT**CAGCTCAAAAC**ACTTAC**
SPArrah/04	3965	14-3931	41.13	1305	150.2	TGA	**GTTTAT**TTGAGCG	TTAGAACTAGCACCC**CA**CAGCTCAAAAC**ACTTAC**
RSArrah/05	3965	14-3931	41.11	1305	150.4	TGA	**GTTTAT**TTGAGCG	TTAGAACTAGCACCC**CT**CAGCTCAAAAC**ACTTAC**
RSArrah/06	3965	14-3931	40.50	1305	150.2	TGA	**GTTTAT**TTGAGCG	TTAGAACTAGCACCC**CT**CAGCTCAAAAC**ACTTAC**
KENrrah/07	3965	14-3931	41.36	1305	150.1	TGA	**GTTTAT**TTGAGCG	TTAGAACTAGCACCC**CA**CAGCTCAAAAC**ACTTAC**
RSArrah/08	3965	14-3931	40.73	1305	150.3	TGA	**GTTTAT**TTGAGCG	TTAGAACTAGCACCC**CT**CAGCTCAAAAC**ACTTAC**
PAKrrah/09	3965	14-3931	41.24	1305	150.2	TGA	**GTTTAT**TTGAGCG	TTAGAACTAGCACCC**TT**CAGCTCAAAAC**ACTTAC**

Terminal hexanucleotides are shown in bold face. Differences in nucleotide sequences are highlighted in dark blue. NCR stands for non-coding regions.

Table 3.2: Percentage identities between AHSV Seg-1 nucleotide and VP1 amino acid sequences.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	**99.9**	99.0	**99.9**	98.9	98.2	99.8	98.6	99.0
RSArrah/02	99.1	ID	98.9	99.8	98.9	98.2	99.7	98.5	98.9
RSArrah/03	94.9	95.5	ID	98.9	98.5	97.9	98.9	98.1	98.5
SPArrah/04	**99.6**	99.2	95.0	ID	98.9	98.2	99.7	98.5	98.9
RSArrah/05	89.9	89.9	89.7	89.9	ID	98.5	98.7	99.4	98.2
RSArrah/06	89.9	90.0	89.8	89.9	95.7	ID	98.0	98.5	**97.6**
KENrrah/07	98.9	98.7	95.4	99.1	89.8	89.8	ID	98.4	98.9
RSArrah/08	90.1	90.1	89.7	90.1	97.8	95.5	90.0	ID	98.0
PAKrrah/09	92.2	92.4	92.2	92.3	**89.1**	89.4	92.2	**89.1**	ID

The analyses were performed on Seg-1 of the nine reference strains of AHSV with nucleotide (white area) and amino acid (blue shaded area) sequences. Symbol "ID" stands for comparison of two identical sequences. Figures in bold highlight values of minimum percentage identity between strains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

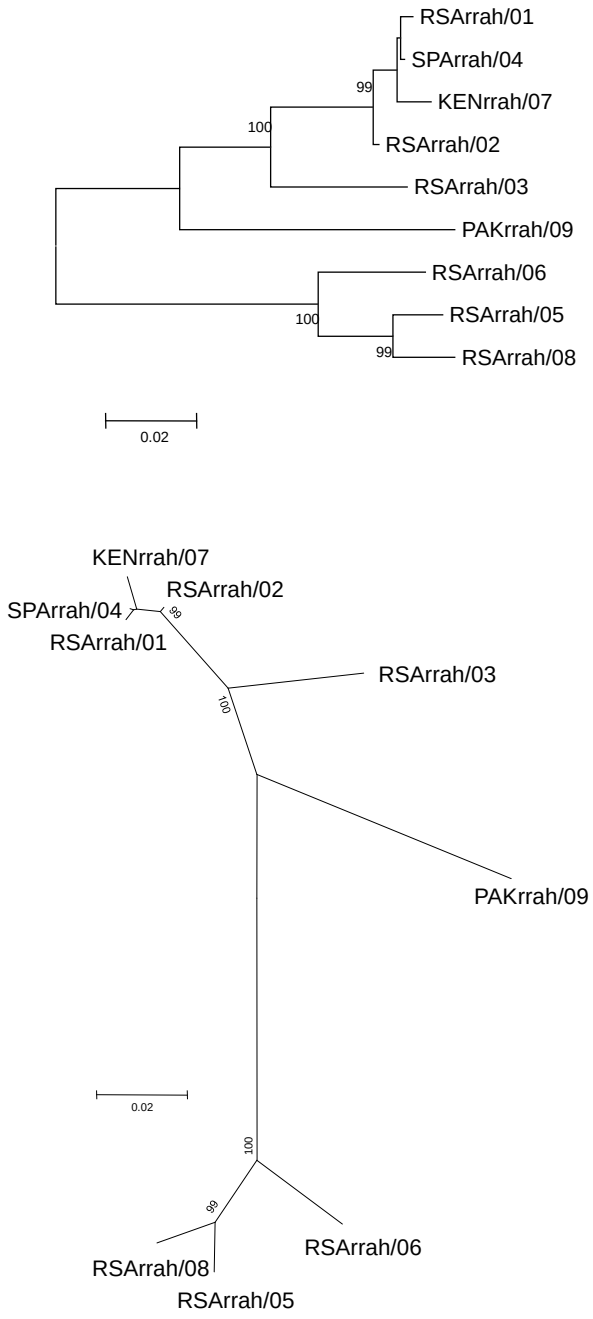


Figure 3.4: Phylogenetic analysis of Seg-1 nucleotide sequence.
The molecular phylogenetic analysis of nucleotide sequence of nine reference strains of AHSV are presented as rectangular (top) and radial (bottom) trees. The analysis was inferred using Maximum likelihood (ML) methods based on the Tamura-Nei model (TN93)(Tamura & Nei, 1993) with a discrete Gamma distribution. The tree is drawn to scale, with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

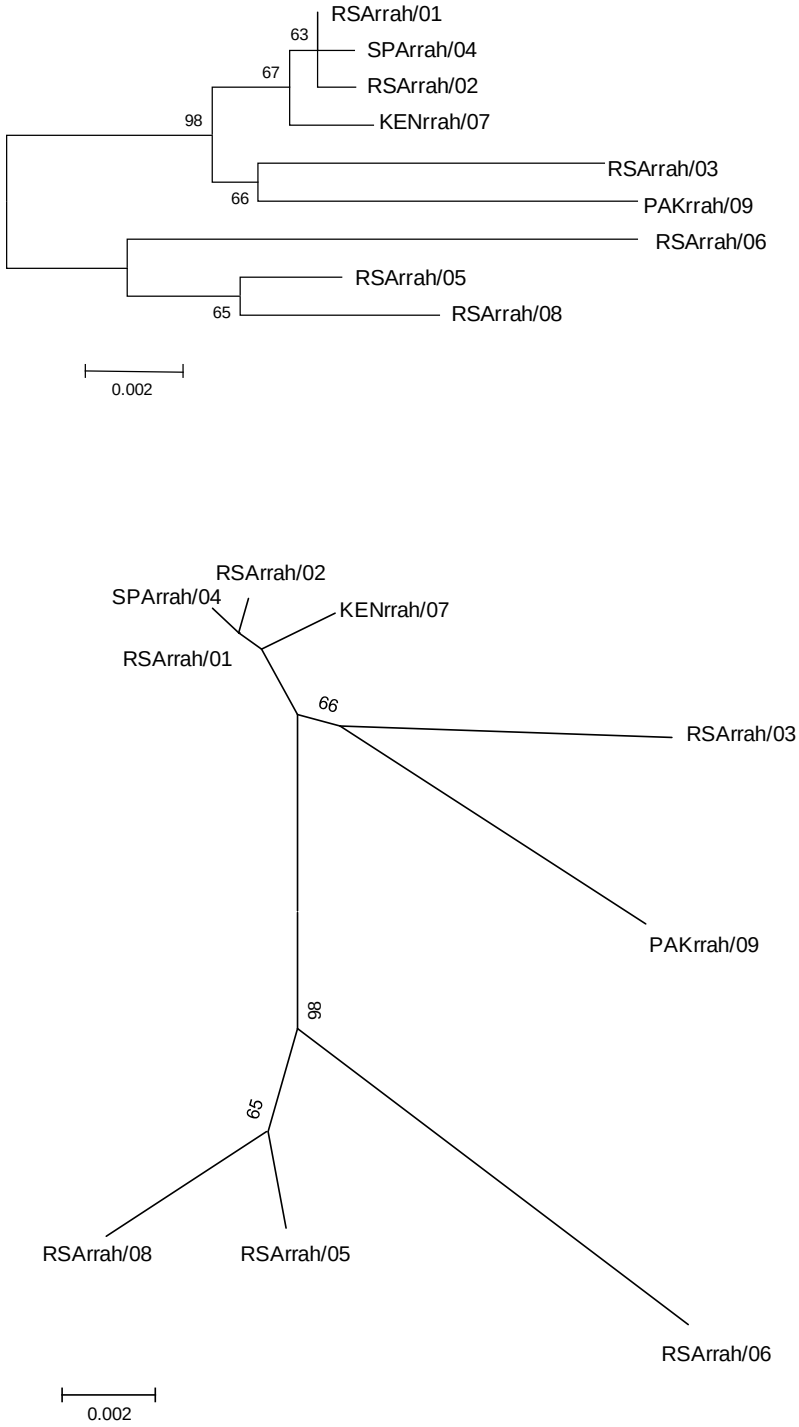


Figure 3.5: Phylogenetic analysis of VP1 amino acid sequence. The molecular phylogenetic analysis of amino acid sequences of nine reference strains of AHSV are presented as rectangular (top) and radial (bottom) trees. The analysis was inferred by using ML method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992). The tree is drawn to scale, with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

3.2.3.2: Genome segment 2

AHSV genome segment 2 (Seg-2) is the most variable of all AHSV segments (particularly between serotypes) and determines the serotype identity of the virus (Bremer *et al.*, 1990). VP2 is largely non-cross reactive between serotypes and does not therefore contribute significantly to the serogroup identity of each strain. This variability also applies to the length of the Seg-2 which differs between 3202 and 3229 nucleotides among the nine reference strains investigated. Some pairs of isolates (serotypes) have identical length Seg-2 (Seg-2 of AHSV-6 and 9 is 3202 nt; AHSV-5 and 8 is 32017 nt; AHSV-1 and 2 is 3218 nt; AHSV-3 and 7 is 3221 nt. Seg-2 of AHSV 4 is the largest at 3229 nt (Table 3.3).

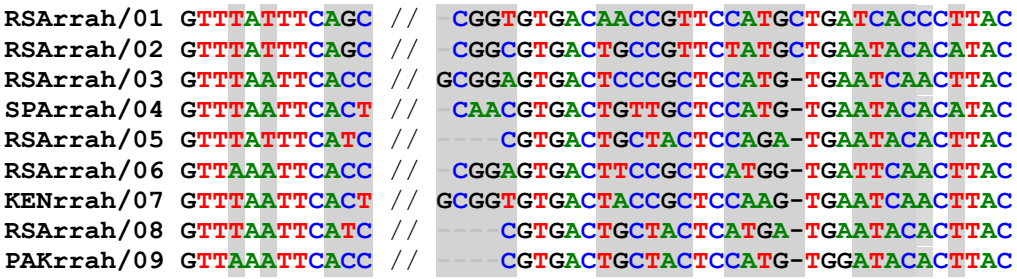


Figure 3.6: Alignment of the 5' and 3' non-coding regions (NCR) of AHSV Seg-2. The open reading frame (ORF) has been removed and replaced with "//". Highlighted in grey are variable regions between all the strains investigated. Figure prepared in BioEdit (Hall, 1999).

Differences in the length of Seg-2 are the result of nucleotide insertions / deletions in both the coding and non-coding regions of the segment. The upstream 5' NCR is the same length (12 nucleotides) in all of the isolates investigated, with both variable and conserved bases and >75% overall identity (Figure 3.6). Unlike Seg-1 the upstream 5' terminal-hexanucleotide is not fully conserved in the fourth and the sixth bases (5'-

GTT$^{T}/_{A}$A$^{T}/_{A}$…3') (Table 3.6). The downstream (3') NCR of Seg-2 varies in length from 35 to 31 nucleotides. It has stretches of bases that are identical in all of the isolates investigated, but only >70% identity overall (Figure 3.6). The 3' terminal hexanucleotide of Seg-2 is also conserved across the reference strains investigated, with two ambiguous sites; its consensus sequence can be presented as 5'... $^{A}/_{C}$C$^{T}/_{A}$TAC-3' (Table 3.3).

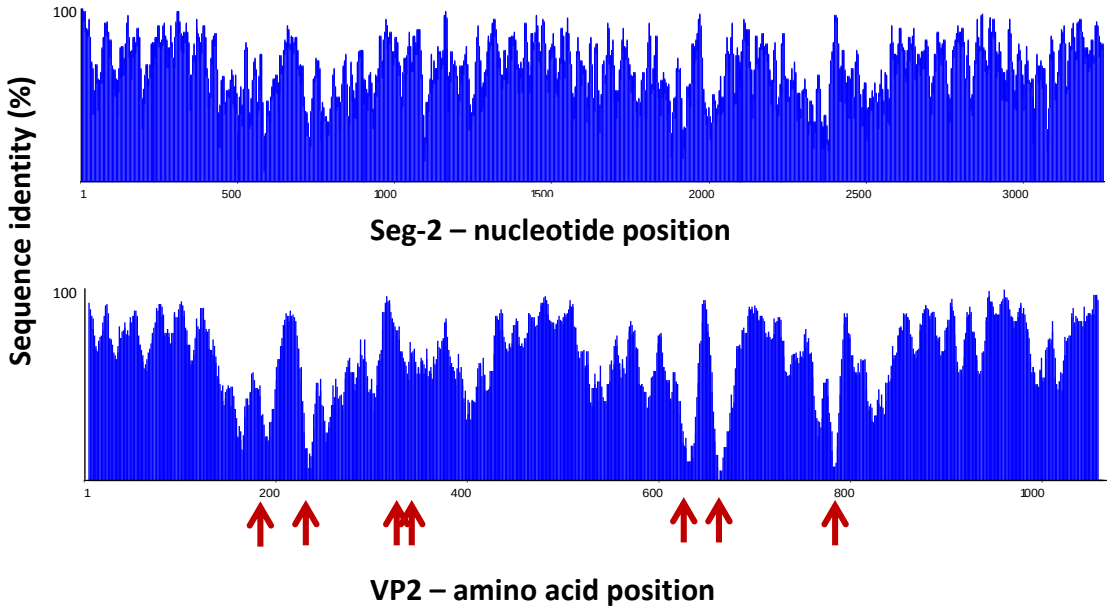


Figure 3.7 Graphical representation of nucleotide and amino acid sequences identities of Seg-2/VP2.
Nucleotide variability is evenly distributed along the sequence of Seg-2. However, the aa sequences of VP2 show more conserved regions interrupted by regions of dramatic variability. Red arrows indicate regions of multiple insertions / deletions events in at least some strains, as illustrated in Figures 3.8 and 3.9. Alignments and plots were constructed using VectorNTI software (Invitrogen); analytical window size 10.

The stop codons used on Seg-2 are not conserved, with RSArrah/01, RSArrah/02, RSArrah/03 and KENrrah/07 employing TAA, while the remaining serotypes use TAG (Table 3.3). Seg-2 shows as little as 55.7% identities, between RSArrah/03 and RSArrah/08 (Table 3.4). However, the following serotypes in pairs display higher levels

of identity: RSArrah/01 and RSArrah/02 (68.2%); RSArrah/03 and KENrrah/07 (66.2%); RSArrah/05 and RSArrah/08 (68.2%); and RSArrah/06 and PAKrrah/09 (63.2%). SPArrah/04 does not appear to be closely related to any of the serotypes investigated, with nucleotide identities between 56.6-58.6% with the remaining reference strains (Table 3.4). The sequence variations between strains are distributed along the whole length of Seg-2, with some areas of increased variability (Figure 3.7). The G + C content also varies between isolates ranging from 39.4 to 41.1% (Table 3.3).

AHSV Seg-2 codes for VP2, the outermost capsid protein, from a single ORF, which differs in length from 3156 to 3183 nucleotides. The resulting proteins therefore fluctuate in length from 1051 to 1060 amino acid, with predicted molecular masses of 122.3-124 kDa (Table 3.3). When the VP2 amino acid sequences were aligned for the nine different AHSV serotypes, it was evident that insertion / deletion events tend to accumulate in several major groupings throughout the alignment. In most cases the "gaps patterns" of deletions and insertions in VP2, group isolates in a similar manner to the sizes of their nucleotide sequences, with similar or identical patterns for the pairs: RSArrah/01 and RSArrah/02; RSArrah/03 and KENrrah/07; RSArrah/05 and RSArrah/08; RSArrah/06 and PAKrrah/09. The longest aa sequence was for VP2 from SPArrah/04, which appears to have fewer deletions compared to the other serotypes (Figure 3.8).

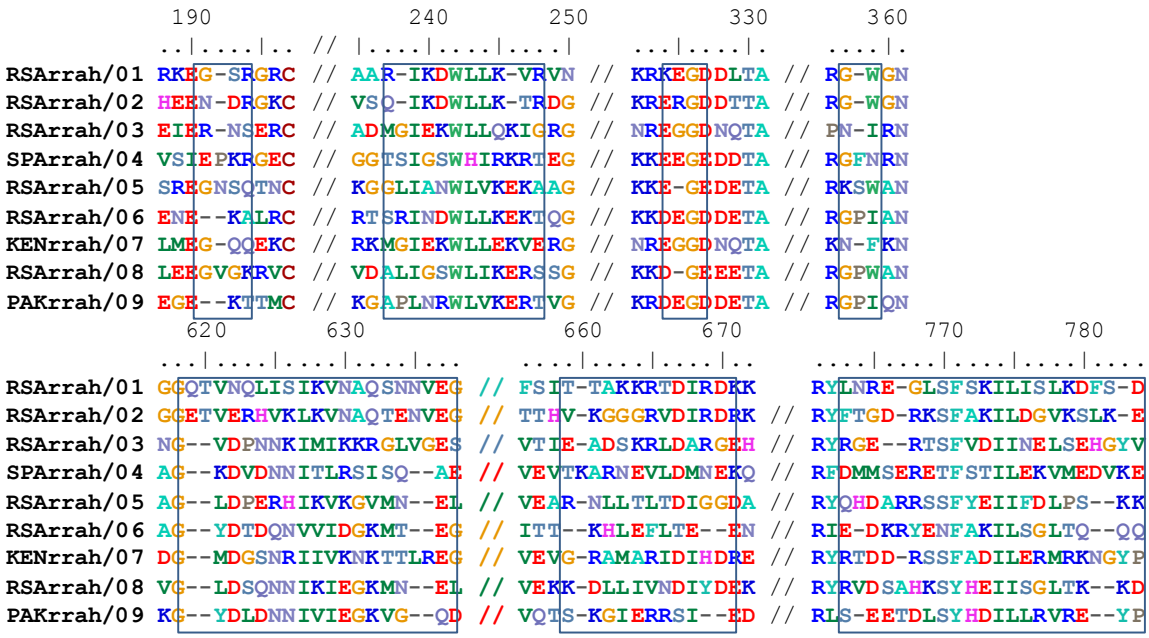


Figure 3.8: Interrupted alignment of VP2 illustrating regions of accumulated insertion / deletion events.
Amino acid alignments were analysed in BioEdit (Hall, 1999); amino acid sequences removed from the alignments are replaced with "//" symbols and the insertion / deletion regions are boxed. Insertions / deletions appear in similar patterns as serotype pairs: RSArrah/01 and RSArrah/02, RSArrah/03 and KENrrah/07, RSArrah/05 and RSArrah/08, RSArrah/06 and PAKrrah/09. Amino acid sequence of SPArrah/04 and this isolate has the least deletions.

The hydrophobicity profile shows that VP2 as largely hydrophilic, with some clear hydrophobic regions (Figure 3.9). When the insertion / deletions (gaps patterns) were overlaid onto the VP2 hydrophobicity plot, in most cases they were located in hydrophilic regions of the protein (Figure 3.9) that are more likely to be surface exposed. This suggests that these differences may be involved in serological differences between serotypes (e.g. neutralisation epitopes). Interestingly, most regions with the highest overall level of diversity detected between the individual strains, overlap with the insertion / deletions (gaps patterns) (Figures 3.7 and 3.8).

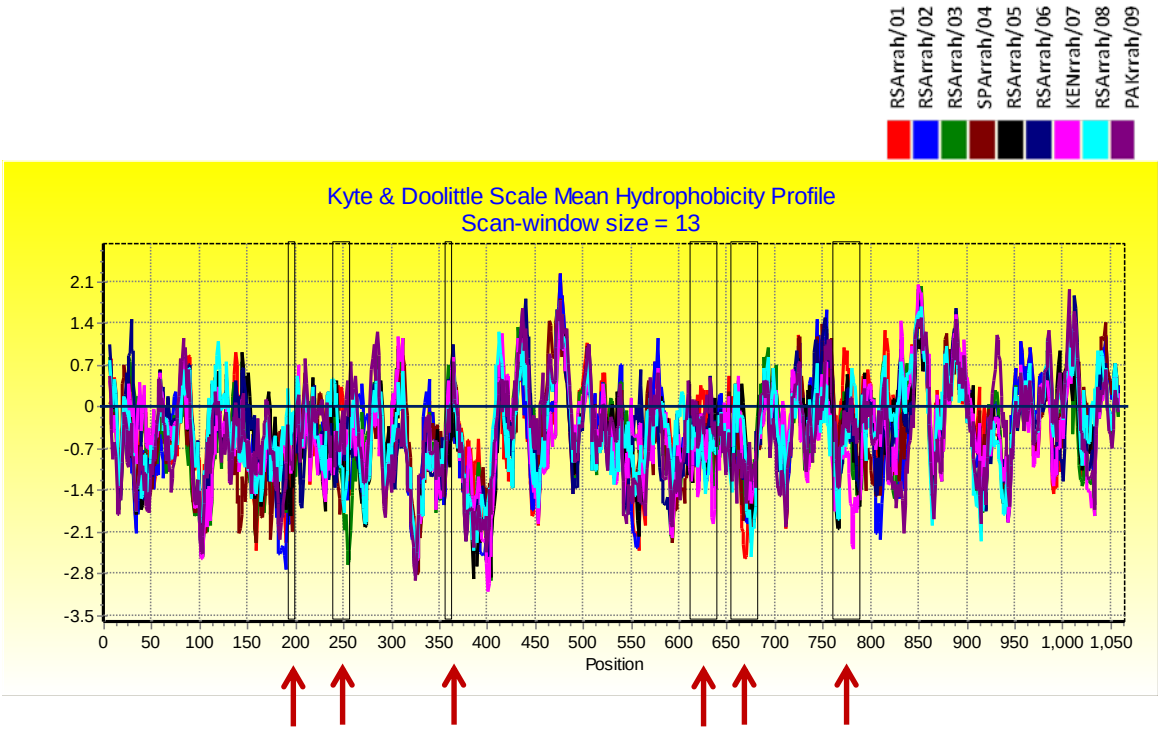


Figure 3.9: Hydrophobicity plot of the VP2 protein.
The plot was inferred using the Kyte and Doolittle Hydrophobicity Scale (Kyte & Doolittle, 1982) accessed through BoiEdit (Hall, 1999). VP2 is mainly hydrophilic in character with hydrophobic regions throughout the sequences. Arrows indicate the regions containing insertions / deletions in multiple serotypes within the amino acid sequences (boxed regions). Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0. Strains of different AHSV serotypes are represented by different coloured lines, as per key.

Percentage identities of the VP2 aa sequence were calculated to be in the range of 49.3 to 72.3% (Table 3.4). This range, wider than that calculated for the percentage identities at the nucleotide level (55.7-68.2%), indicates that evolutionary events involving both synonymous and non-synonymous events are taking place in the protein coding sequence of Seg-2. The ratio of non-synonymous (d_N) to synonymous (d_S) substitutions per site was estimated to be $d_N/d_S = 0.317$, which indicates overall purifying selection ($d_N/d_S < 1$) on the protein coding sequence. The nucleotide substitution rate of Seg-2 is significantly

higher than that of Seg-1, at 1.96 substitutions per site for the VP2 coding sequences analysed in this study (Table 3.22).

The ML phylogenetic analysis (Felsenstein, 1981) of both, the nucleotide and amino acid sequences reveals similar topology where isolates of all nine reference strains investigated, grouped separately and with a clear distance between them (Figures 3.10 and 3.11). Despite the clear separation of the Seg-2 sequences for each of the nine reference strains / serotypes, some pairs of strains are more closely related: RSArrah/01 and RSArrah/02; RSArrah/03 and KENrrah/07; RSArrah/05 and RSArrah/08; RSArrah/06 and PAKrrah/09. SPArrah/04 (AHSV-4) did not group closely with any of the other strains. This pattern of relationships is consistent with the 'gaps pattern' and the sizes of Seg-2/VP2 (see above). For comparison purposes phylogenetic trees were also constructed using the NJ method (Saitou & Nei, 1987) with very similar results (data not shown).

Table 3.3: Genetic information for Seg-2/VP2 of AHSV-1 to 9 reference strains.

AHSV isolate name	Segment length (bp)	Open reading frame (bp)	G+C content (%)	Protein size (aa)	Predicted protein mol mass (KDa)	Stop codon	5' Terminal sequence	3' Terminal sequence
RSArrah/01	3218	13-3183	40.7	1056	123.2	TAA	GTTTATT...	...CCTTAC
RSArrah/02	2318	13-3183	40.6	1056	123.2	TAA	GTTTATT...	...ACATAC
RSArrah/03	3221	13-3186	41.1	1057	123.1	TAA	GTTTAAT...	...ACTTAC
SPArrah/04	3229	13-3195	40.1	1060	124	TAG	GTTTAAT...	...ACATAC
RSArrah/05	3217	13-3186	40.3	1057	122.8	TAG	GTTTATT...	...ACTTAC
RSArrah/06	3202	13-3168	40.8	1051	122.3	TAG	GTTAAAT...	...ACTTAC
KENrrah/07	3221	13-3186	39.9	1057	123.5	TAA	GTTTAAT...	...ACTTAC
RSArrah/08	3217	13-3186	39.4	1057	123.3	TAG	GTTTAAT...	...ACTTAC
PAKrrah/09	3202	13-3171	39.8	1052	123.4	TAG	GTTAAAT...	...ACTTAC

Differences in nucleotide sequences are highlighted in dark blue.

Table 3.4: Percentage identities between Seg-2 nucleotide and VP2 amino acid sequences.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	**72.3**	53.1	51.8	51.3	51.2	54.3	50.7	50.7
RSArrah/02	**68.2**	ID	53.7	52.2	51.8	49.6	54.7	51.2	**49.3**
RSArrah/03	58.3	57.8	ID	51.8	51.4	50.3	66.1	50.9	51.0
SPArrah/04	57.7	56.8	56.7	ID	53.4	51.5	50.6	52.1	51.9
RSArrah/05	56.3	56.0	56.0	58.6	ID	53.5	51.3	70.9	52.4
RSArrah/06	56.7	56.3	56.1	57.5	58.2	ID	51.5	52.0	62.5
KENrrah/07	58.4	58.8	66.2	56.6	57.4	57.6	ID	51.4	52.0
RSArrah/08	56.3	56.4	**55.7**	57.6	**68.2**	57.5	56.6	ID	53.1
PAKrrah/09	56.8	56.6	56.1	58.5	57.7	63.2	56.0	59.0	ID

Seg-2/VP2 of the nine reference strains of AHSV were compared, showing nucleotide (white area) and amino acid (blue shaded area) sequences. Symbol "ID" stands for comparison of two identical sequences. Figures in bold highlight values of minimum percentage identity between strains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

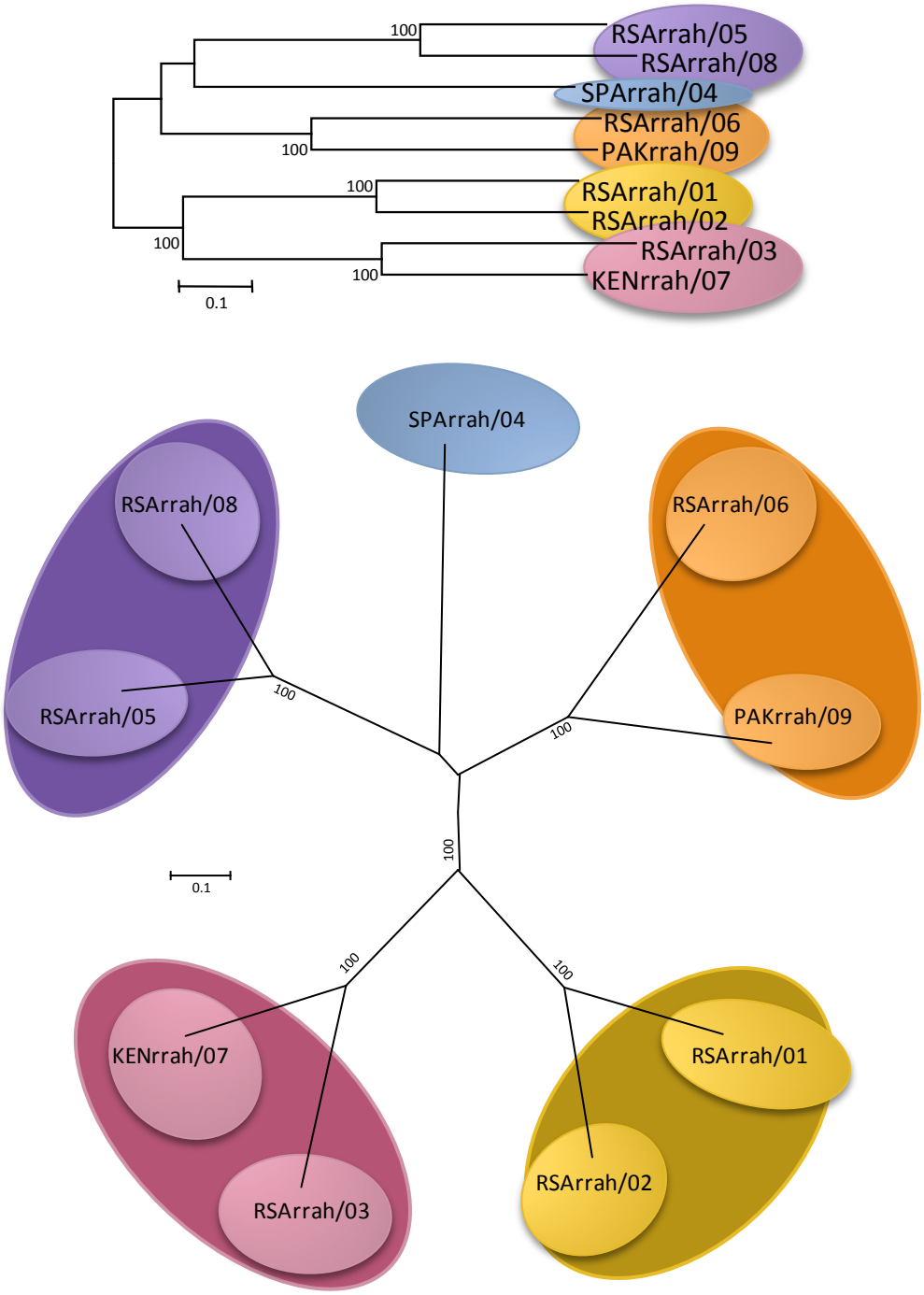


Figure 3.10: Phylogenetic analysis of Seg-2 nucleotide sequences.
The phylogenetic analysis of nucleotide sequence of Seg-2 of the nine reference strains of AHSV are presented as rectangular (top) and radial (bottom) trees. The analysis was inferred using a ML method, based on the General Time Reversible (GTR) model with a discrete Gamma distribution. The rate variation model allowed for some sites to be evolutionary invariable. The tree is drawn to scale, with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura et al., 2011).

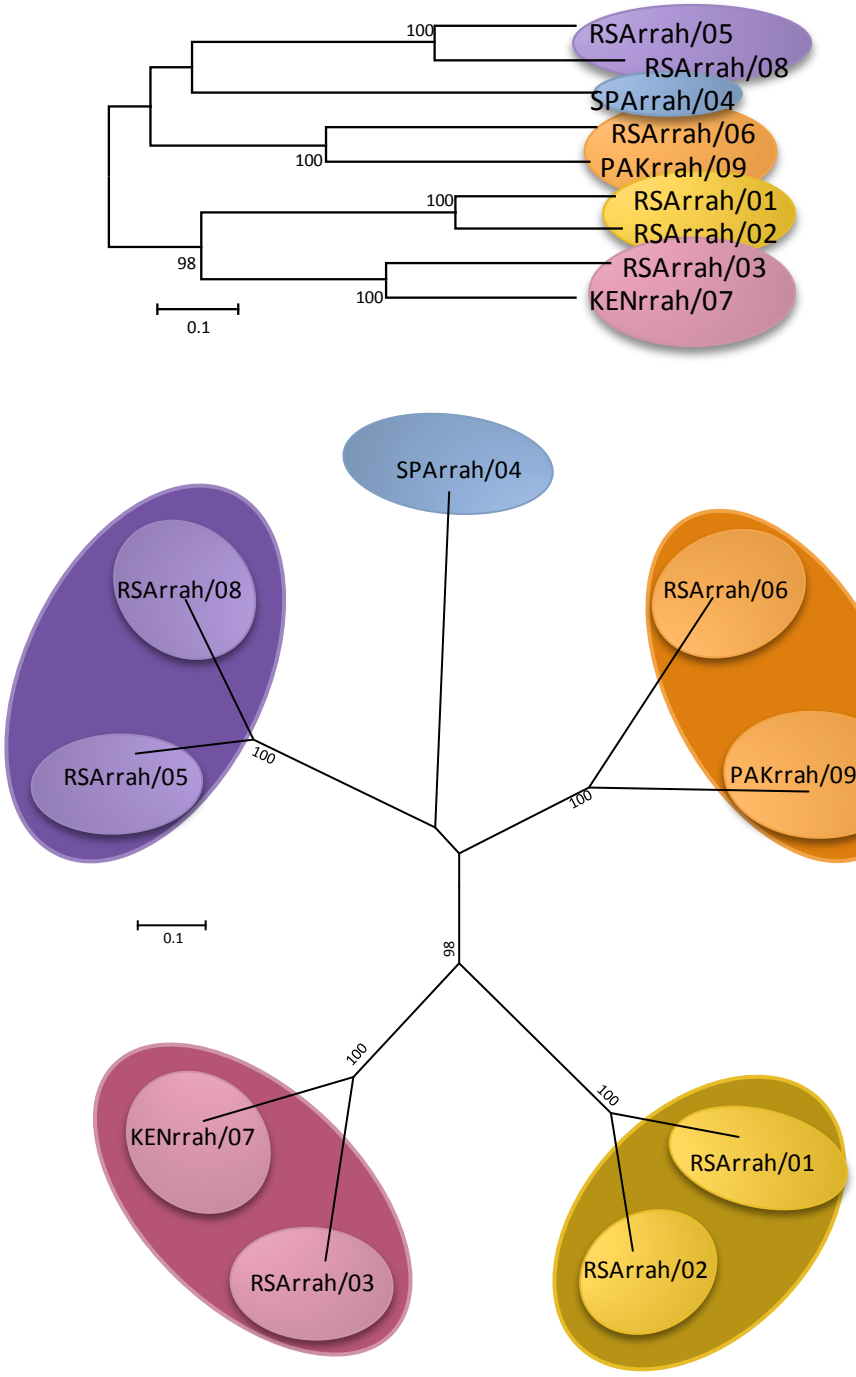


Figure 3.11: Phylogenetic analysis of VP2 amino acid sequence.

Phylogenetic analysis of the aa sequence of VP2 from the nine reference strains of AHSV are presented as rectangular (top) and radial (bottom) trees. The analysis was inferred by using ML method based on the Whelan and Goldman + Freq. model (WAG+F) (Whelan and Goldman, 2001) with a discrete Gamma distribution. The rate variation model allowed for some sites to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura et al., 2011).

3.2.3.3: Genome segment 3

Seg-3 is the most conserved genome segment of the AHSV strains analysed, at 2792 nucleotides in length. Both 5' and 3' NCR of the segment are identical in all of the reference strains, and are 26 or 48 nucleotides in length respectively. The terminal hexanucleotides are therefore also maintained across every strain investigated as 5'-GTTTAT...ACTTAC-3'. TGA is used to terminate the VP3 ORF in all of the reference strains (Table 3.5). The overall nt identity level in Seg-3 is even higher than Seg-1, at >95.5% for the strains investigated. Three reference strains (SPArrah/04, RSArrah/05 and RSArrah/08) have identical versions of Seg-3 (Table 3.6), suggesting a common ancestry and / or reassortment during the evolution of these strains. The small numbers of nt differences between the strains are distributed along the length of Seg-3, with some regions of complete conservation between all of the strains (Figure 3.12).

Seg-3 of AHSV encodes the sub-core protein VP3 from a single large ORF, positioned between nucleotides 27-2744. VP3 is 905 aa in length and has an identical molecular weight for all of the reference strains at 103.2 kDa (Table 3.5). VP3 is the most conserved AHSV protein with > 99% identity between the different strains analysed (Table 3.6). Consequently the VP3 hydrophobicity profiles are almost identical, showing regions of both hydrophobic and hydrophilic nature (Figure 3.13). The high levels of aa identity in VP3 are illustrated by sequence alignments (Figure 3.12); showing only occasional differences in the N-terminal and central part of the protein. Six out of nine reference strains (RSArrah/01, RSArrah/02, SPArrah/04, RSArrah/05, KENrrah/07, RSArrah/08) have VP3 with the same amino acid sequence (Table 3.6), which reflects the importance and conservation of this 'T2' protein as a primary determinant of the overall size and organisation of the virus particle structure (see Chapter 1, section 1.3). This conclusion is

supported by the low substitution rate of 3.69 x 10^2 per site for the VP3 coding sequence typical of very strong purifying selection on the protein coding sequence, with a ratio of non-synonymous (d_N) to synonymous (d_S) substitutions per site (d_N/d_S) of 0.012 (Table 3.22).

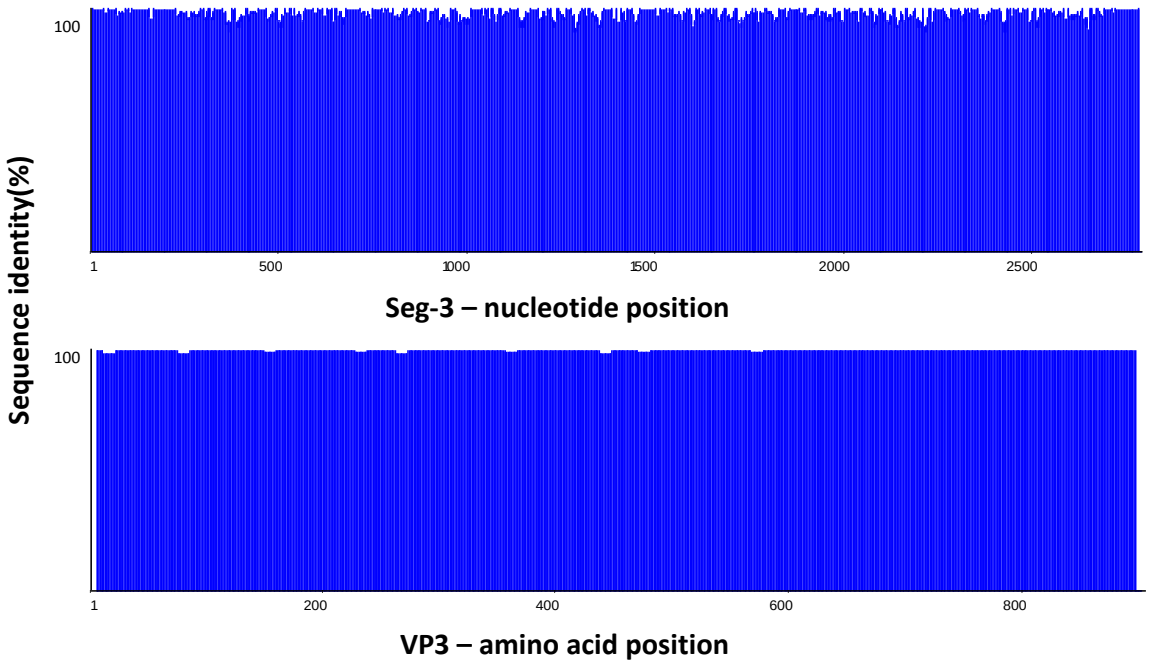


Figure 3.12: Graphical representation of nucleotide and amino acid sequences identities of Seg-3 / VP3.
High level percentage identity within nucleotide sequence of Seg-3 between nine reference stain of AHSV hardly translates to amino acid differences between the strains analyses. Alignments and plots were constructed using VectorNTI software (Invitrogen); analytical window size 10.

Phylogenetic analysis, by ML methods (Felsenstein, 1981), confirmed the close relationship between AHSV strains (Figure 3.14 and 3.15). The comparative scale for the nucleotide sequence analysis of 0.005 nucleotide substitutions per site for all nine reference strains again indicates a very close overall relationship within this segment.

Even finer comparative scale of 0.0005 amino acid substitutions per site was used to show the relationship between the aa sequences of VP3. The trees show that Seg-3 nt sequences of the three remaining types (RSArrah/03, RSArrah/06 and PAKrrah/09) vary only fractionally (≥ 99.2% identity) from the other identical aa sequences of the reference strains. NJ (Saitou & Nei, 1987) trees for this dataset were also drawn for comparison purposes and displayed similar topology (data not shown).

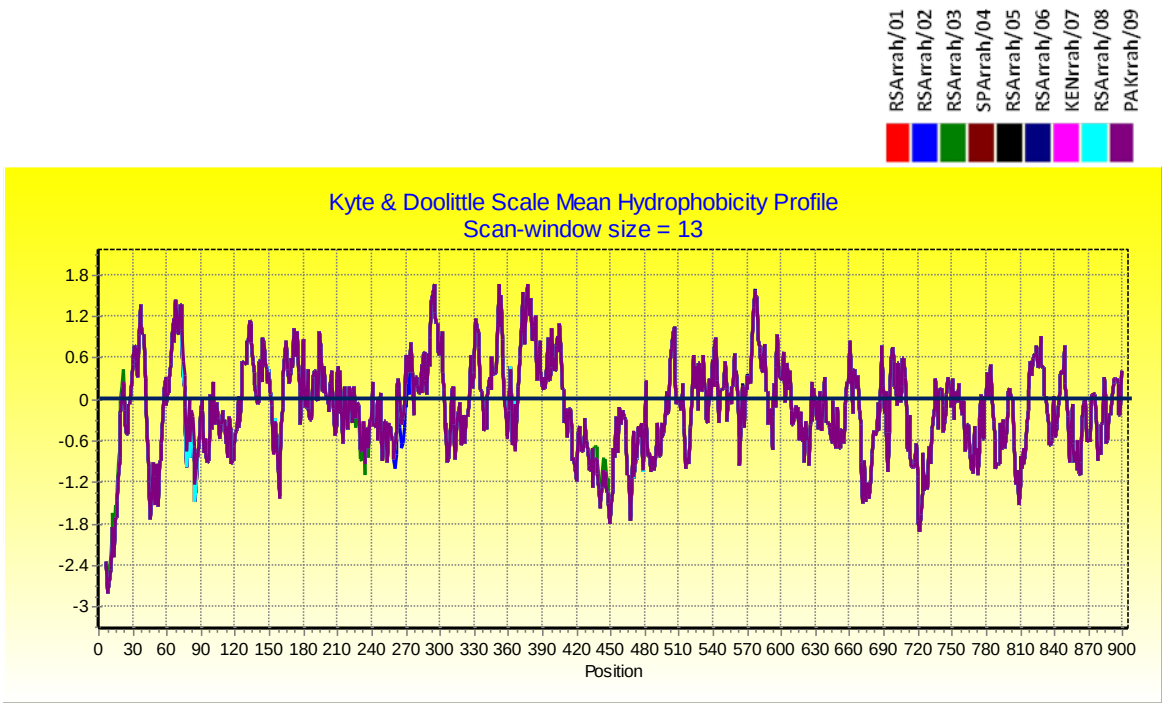


Figure 3.13: Hydrophobicity plot of the VP3 protein.

The plot was inferred using Kyte and Doolittle Hydrophobicity Scale (Kyte and Doolittle, 1982) accessed through BioEdit (Hall, 1999). The analyses demonstrate regions of both hydrophobic and hydrophilic nature of the protein. Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0. Strains of different AHSV serotypes are represented by different coloured lines, as per key.

Table 3.5: Genetic information for Seg-3 /VP3 of the nine AHSV reference strains.

AHSV isolate name	Segment length (bp)	Open reading frame (bp)	G+C content (%)	Protein size (aa)	Predicted protein mol.weight (kDA)	Stop codon	5' Terminal sequence	3' Terminal sequence
RSArrah/01	2792	27-2744	43	905	103.2	TGA	GTTTAT…	...ACTTAC
RSArrah/02	2792	27-2744	43	905	103.2	TGA	GTTTAT…	...ACTTAC
RSArrah/03	2792	27-2744	43	905	103.2	TGA	GTTTAT…	...ACTTAC
SPArrah/04	2792	27-2744	42.9	905	103.2	TGA	GTTTAT…	...ACTTAC
RSArrah/05	2792	27-2744	42.9	905	103.2	TGA	GTTTAT…	...ACTTAC
RSArrah/06	2792	27-2744	43.3	905	103.2	TGA	GTTTAT…	...ACTTAC
KENrrah/07	2792	27-2744	43.1	905	103.2	TGA	GTTTAT…	...ACTTAC
RSArrah/08	2792	27-2744	42.9	905	103.2	TGA	GTTTAT…	...ACTTAC
PAKrrah/09	2792	27-2744	43.1	905	103.2	TGA	GTTTAT…	...ACTTAC

Table 3.6: Percentage identities between Seg-3 nucleotide and VP3 amino acid sequences.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	**100**	99.7	**100**	**100**	99.9	**100**	**100**	99.6
RSArrah/02	97	ID	99.7	**100**	**100**	99.9	**100**	**100**	99.6
RSArrah/03	96.7	98.6	ID	99.7	99.7	99.6	99.7	99.7	**99.2**
SPArrah/04	96.8	97.5	97.2	ID	**100**	99.9	**100**	**100**	99.6
RSArrah/05	96.8	97.5	97.2	100	ID	99.9	**100**	**100**	99.6
RSArrah/06	96.1	96.7	96.4	96.3	96.3	ID	99.9	99.9	99.4
KENrrah/07	99.6	97	96.7	96.7	96.7	96.1	ID	**100**	99.6
RSArrah/08	96.8	97.5	97.2	**100**	**100**	96.3	96.7	ID	99.6
PAKrrah/09	95.6	95.7	**95.5**	95.6	95.6	95.9	95.6	95.6	ID

Analyses were performed on Seg-3 of nine reference strains of AHSV and involved nucleotide (white area) and amino acid (blue shaded area) sequences. "ID" stands for comparison of two identical sequences. Figures in bold highlight values of minimum percentage identity between strains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

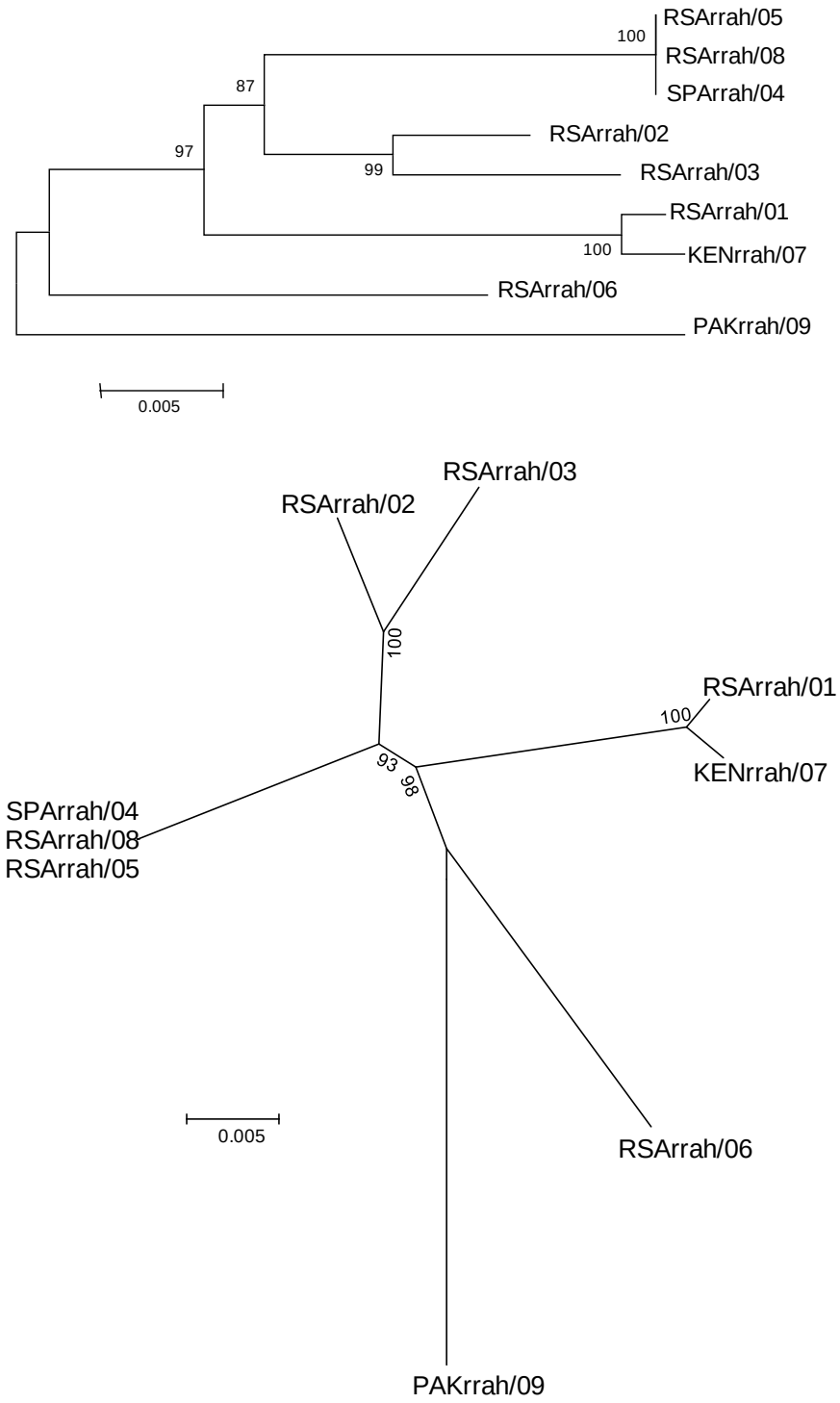


Figure 3.14: Phylogenetic analysis of Seg-3 nucleotide sequences.
Phylogenetic analysis of nucleotide sequence of Seg-3 from the nine reference strains of AHSV are presented as rectangular (top) and radial (bottom) trees. The analysis was inferred using ML method based on Tamura-Nei model (TN93) (Tamura & Nei, 1993) with a discrete Gamma distribution. The tree is drawn, with branch lengths determined by the numbers of substitutions per site, with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

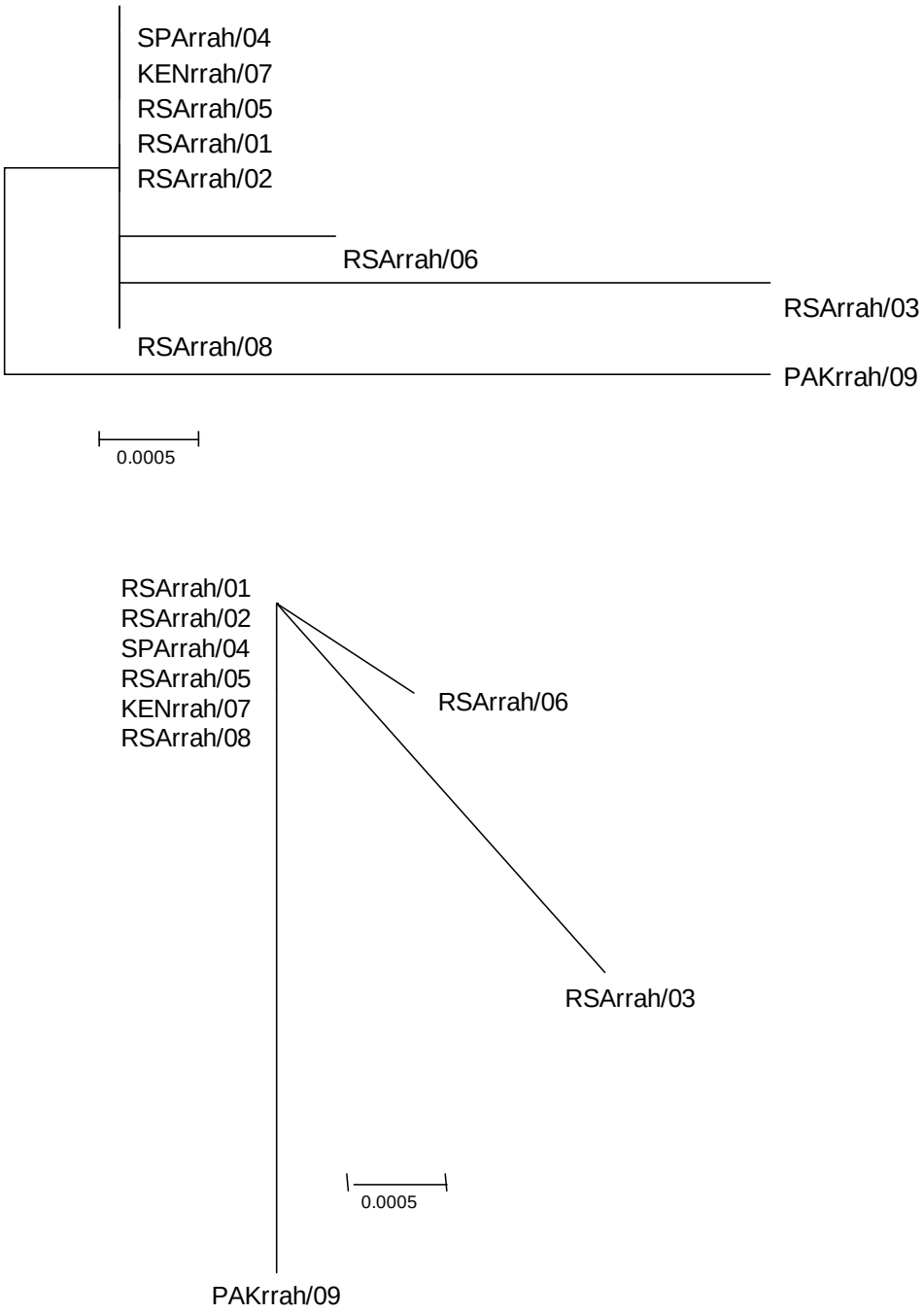


Figure 3.15: Phylogenetic analysis of VP3 amino acid sequences.
Phylogenetic analysis of the aa sequences of VP3 from the nine reference strains of AHSV are presented as rectangular (top) and radial (bottom) trees. The analysis used a ML method based on the General Reversible Chloroplast model (cpREV) (Adachi *et al.*, 2000). The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

3.2.3.4: Genome segment 4

Genome segment 4 (Seg-4) is also highly conserved (at 1978 bp) across all nine reference strains of AHSV. The 5' NCR region is 11 nucleotides long and is conserved between the strains apart from a T to A substitution at the fourth base pair within the 5' hexanucleitide sequences of RSArrah/06 and PAKrrah/09 (Table 3.7). The 5' hexanucleitide consensus for all nine studied reference strains can be summarised as 5'-GTT$^{T}/_{A}$AT…3'. The 3' NCR is significantly longer (38 nt) and is also highly conserved with only one nucleotide change between all of the strains investigated, and identical 3' terminal hexanucleotide sequences (5'...CCTTAC-3' - Table 3.7). The overall level of nt sequence identity in Seg-4 of the AHSV reference strains is > 91% (Table 3.8), with nucleotide variations evenly distributed throughout its length (Figure 3.16) and a G+C content of 42 to 42.7%. Two isolates (RSArrah/04 and RSArrah/05) have exactly the same nucleotide sequence of this genome segment (Table 3.7).

AHSV Seg-4 codes for the minor core protein and capping enzyme VP4, from a single ORF of conserved size (nucleotides 12 to 1940) in all nine reference strains, with the same stop codon 'TAA' in each case. The VP4(CaP) protein is 642 aa in length (Table 3.7) and is predicted to be of mainly of hydrophilic in character but with clear hydrophobic regions (Figure 3.17). The calculated molecular mass for VP4 varies between isolates from 75.6 to 75.8 kDa (Table 3.7). The minimum overall aa identity level is ≥ 95.5% (for RSArrah/03 and RSArrah/06 up to 100% for RSArrah/04 and RSArrah/05). The aa sequence identity levels for VP4, are higher than the nt identity of Seg-4, indicating significant evolutionary constraints on the protein. Conservation of the protein is graphically illustrated in Figure 3.16, which shows only very limited aa differences in VP4 between the nine AHSV reference strains. Calculation of d_N/d_S ratio

(d_N/d_S = 0.07 per site) indicates strong purifying selection on the protein coding sequence (Table 3.22). The nucleotide substitution rate for the protein coding sequence of Seg-4 was estimated to be 6 x 10^{-2} substitution per site (Table 3.22).

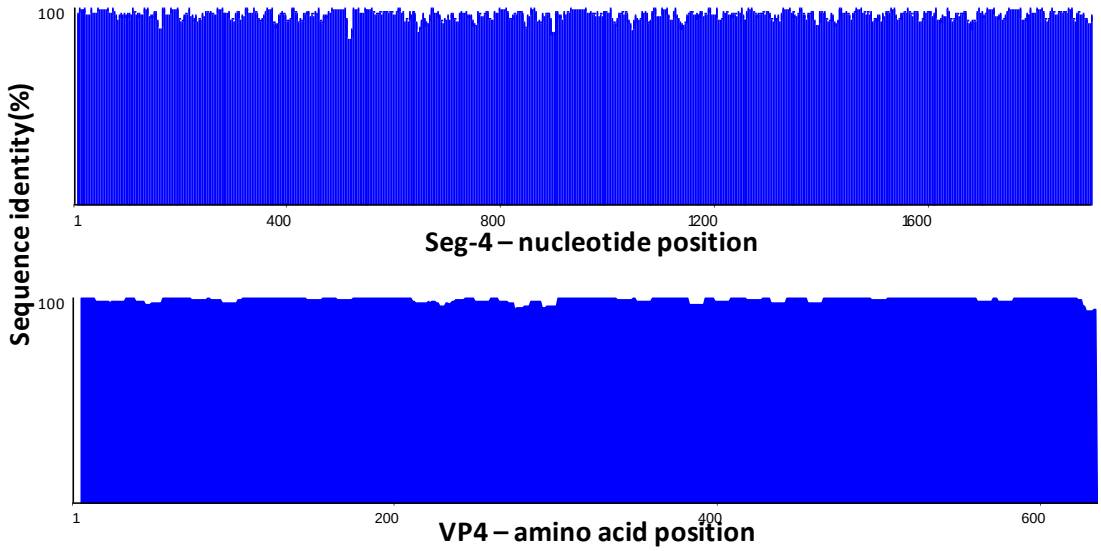


Figure 3.16: Graphical representation of nucleotide and amino acid sequences identities of Seg-4 / VP4.
Low levels of nucleotide variation were detected between the nine reference strains of AHSV, and changes are evenly spread throughout within Seg-4 sequences. These differences translate to a few changes dispersed amino acid changes in VP4, mainly within the central region of the sequence. Alignments and plots were constructed using VectorNTI software (Invitrogen); window size 10.

ML phylogenetic (Felsenstein, 1981) for the nt sequences of Seg-4 show close relationships between the AHSV reference strains, with RSArrah/06 grouping more distantly from the rest of the isolates (Figures 3.18 and 3.19). Phylogenetic analysis of VP4 aa sequences, required a much finer 'comparison scale' of 0.005 substitutions (Figure 3.19), but still showed a similar tree topology. The bootstraps values supporting branching of the amino acid sequence trees are lower than recommended in the literature (Hillis & Bull, 1993), however confidence in the tree is supported by a very similar

phylogenetic topology constructed using NJ method (Saitou & Nei, 1987) (data not shown).

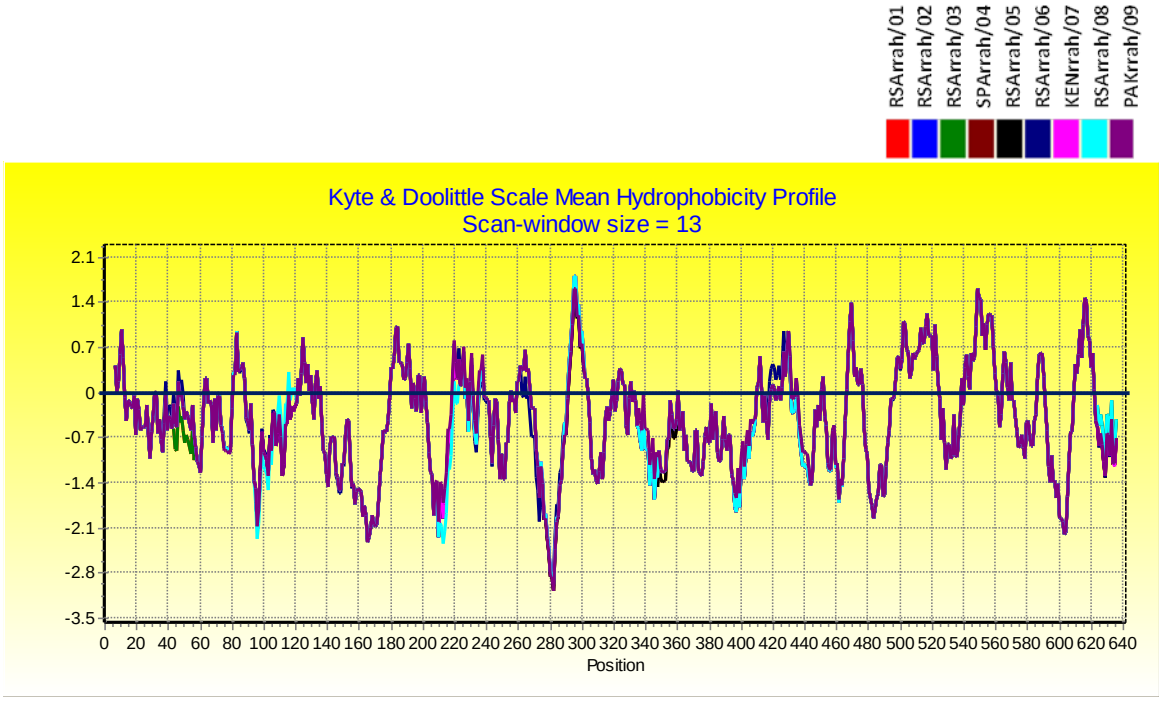


Figure 3.17: Hydrophobicity plot of the VP4 protein.
The plot was inferred using Kyte and Doolittle Hydrophobicity Scale (Kyte and Doolittle, 1982) accessed through BoiEdit (Hall, 1999). The analyses demonstrate mainly hydrophilic character of the protein with sporadic hydrophobic domains throughout the length of the protein. Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0. Strains of different AHSV serotypes are represented by different coloured lines, as per key.

Table 3.7: Genetic information for Seg-4/VP4 of the nine AHSV reference strains.

AHSV isolate name	Segment length (bp)	Open reading frame (bp)	G+C content (%)	Protein size (aa)	Predicted protein molecular mass (kDA)	Stop codon	5' Terminal sequence	3' Terminal sequence
RSArrah/01	1978	12-1940	42	642	75.8	TAA	GTTTAT…	…CCTTAC
RSArrah/02	1978	12-1940	42.1	642	75.7	TAA	GTTTAT…	…CCTTAC
RSArrah/03	1978	12-1940	42	642	75.7	TAA	GTTTAT…	…CCTTAC
SPArrah/04	1978	12-1940	42.3	642	75.8	TAA	GTTTAT…	…CCTTAC
RSArrah/05	1978	12-1940	42.3	642	75.8	TAA	GTTTAT…	…CCTTAC
RSArrah/06	1978	12-1940	42.7	642	75.8	TAA	GTTAAT…	…CCTTAC
KENrrah/07	1978	12-1940	42	642	75.8	TAA	GTTTAT…	…CCTTAC
RSArrah/08	1978	12-1940	42	642	75.6	TAA	GTTTAT…	…CCTTAC
PAKrrah/09	1978	12-1940	42.3	642	75.8	TAA	GTTAAT…	…CCTTAC

Differences in nucleotide sequences are highlighted in dark blue.

Table 3.8: Percentage identities between Seg-4 nucleotide and VP4 amino acid sequences.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	99.1	99.7	99.2	99.2	95.8	98.9	99.1	98.3
RSArrah/02	96.1	ID	99.4	99.4	99.4	96.1	99.2	99.7	98.3
RSArrah/03	99.8	96.1	ID	99.2	99.2	**95.5**	98.9	99.4	98.0
SPArrah/04	95.8	98.9	95.7	ID	**100**	95.8	99.1	99.2	98.3
RSArrah/05	95.8	98.9	95.7	**100**	ID	95.8	99.1	99.2	98.3
RSArrah/06	**91.0**	91.5	**91.0**	**91.0**	**91.0**	ID	96.0	95.8	96.0
KENrrah/07	95.3	98.9	95.3	98.2	98.2	91.1	ID	99.2	98.1
RSArrah/08	96.4	97.8	96.4	96.9	96.9	91.1	96.9	ID	98.0
PAKrrah/09	95.4	96.4	95.2	95.8	95.8	91.4	95.6	96.2	ID

The nt sequences of Seg-4 (white area) and the aa sequences (blue shaded area) of VP4 were compared for the nine AHSV reference strains. "ID" stands for comparison of two identical sequences. Figures in bold highlight values of minimum percentage identity between the trains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

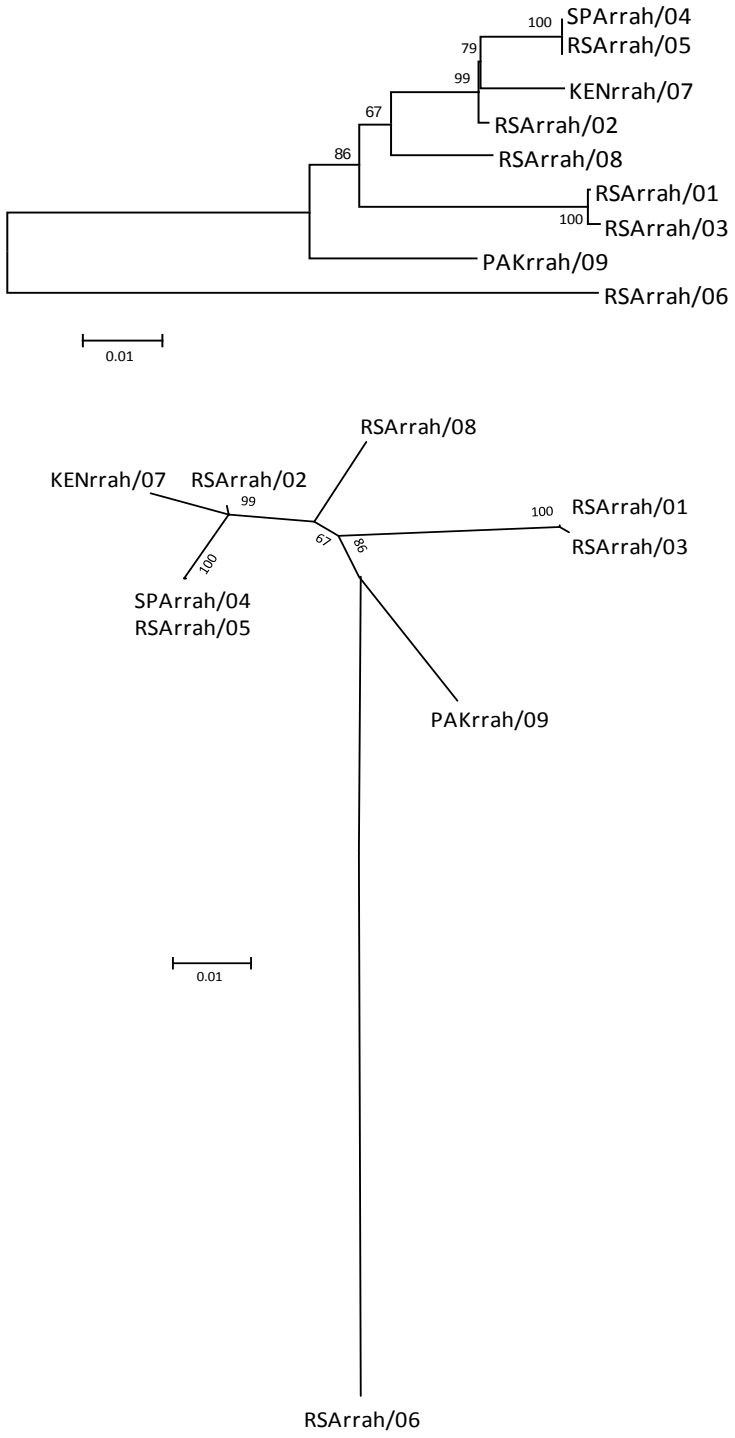


Figure 3.18: Phylogenetic analysis of AHSV Seg-4 nucleotide sequence.
Phylogenetic analyses of nt sequences of Seg-4 from the AHSV reference strains using a ML method based on the Tamura-Nei model (TN93) (Tamura & Nei, 1993) with a discrete Gamma distribution (rectangular (top) and radial trees(bottom)). The rate variation model allowed for some sites to be evolutionarily invariable. The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

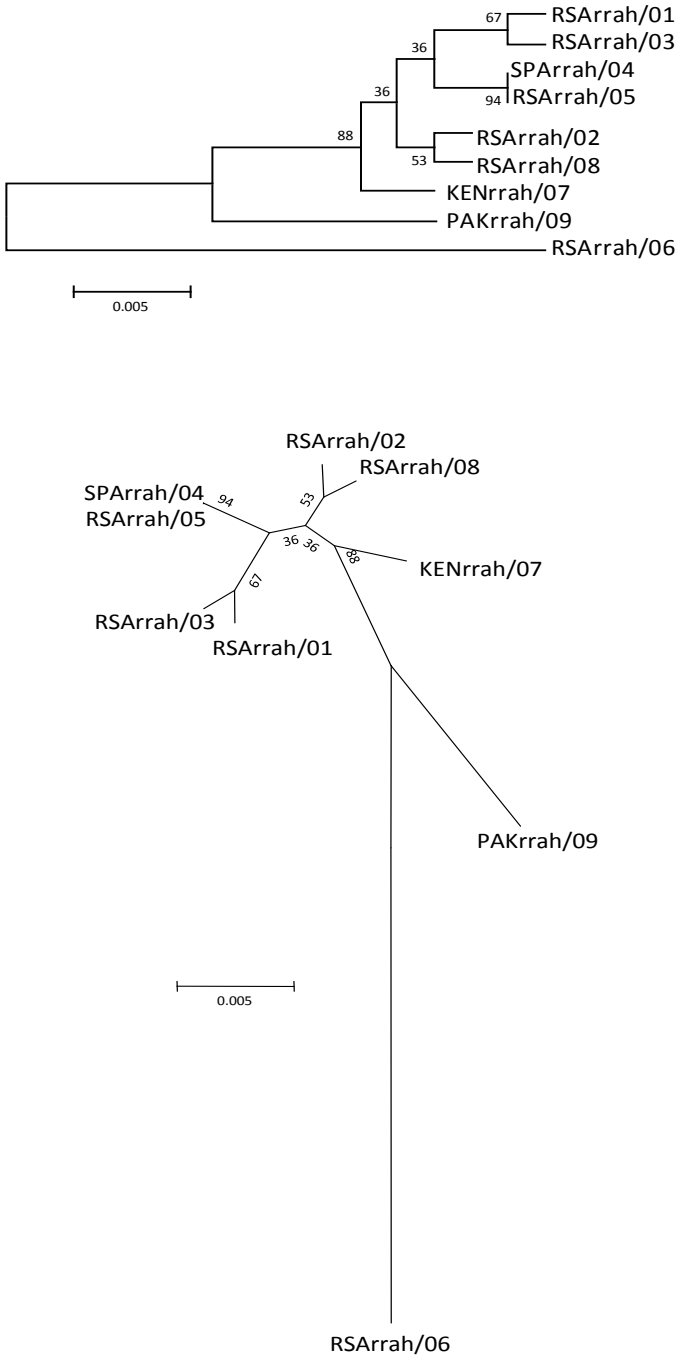


Figure 3.19: Phylogenetic analysis of AHSV VP4 amino acid sequence.
Phylogenetic analysis of aa sequences of VP4 from the nine AHSV reference strains are presented as rectangular (top) and radial (bottom) trees. The analysis uses ML method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992). The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

3.2.3.5: Genome segment 5

The length of Seg-5 (1748 bp) is the same for all nine of the AHSV reference strains investigated. The upstream 5' NCR is 35 nt, and was identical in each case, while the 3' NCR showed >97% identity. Both of the terminal hexanucleotides were fully conserved in Seg-5, as 5'- GTTAAA...ACTTAC – 3' (Table 3.9). The nucleotide sequence identity plots show relatively few and uniformly distributed variations (Figure 3.20). Identity levels in Seg-5 between individual strains range from 95.6% (RSArrah/06 and PAKrrah/09) to 100% (SPArrah/04 and RSArrah/05) (Table 3.10). The G+C content of this segment is between 41.6-41.9% (Table 3.9).

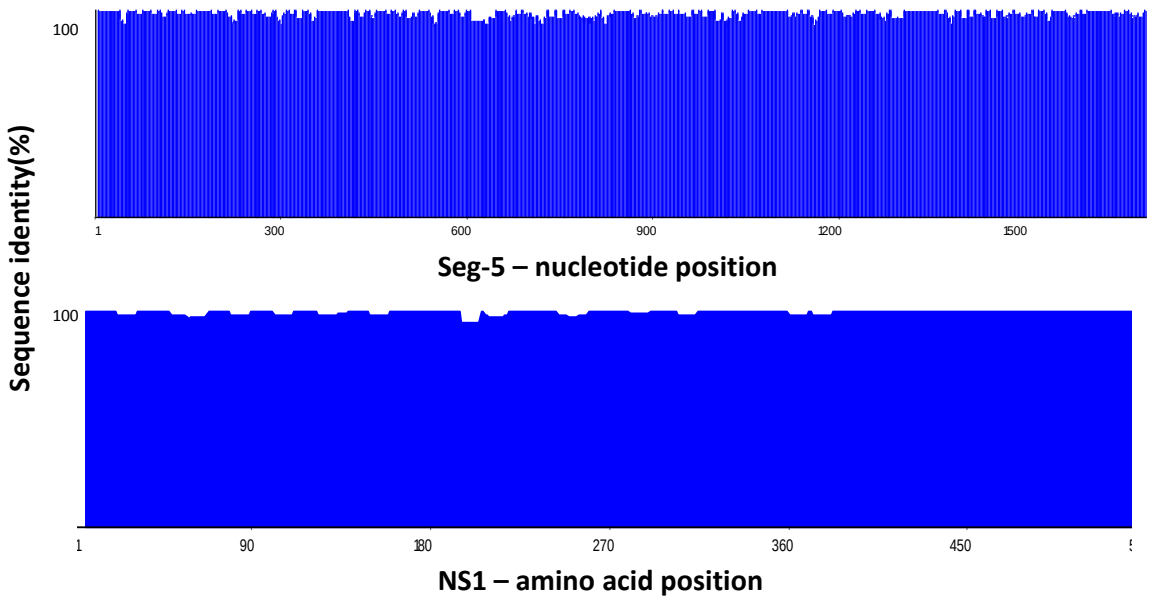


Figure 3.20: Graphical representation of nucleotide and amino acid sequences identities of Seg-5 / NS1.
Low level nt differences between the nine AHSV reference strains are evenly spread throughout Seg-5 sequences. The differences translate to a small number of changes, mainly within N-terminal and central part of the aa sequence of NS1. Alignments and plots were constructed using VectorNTI software (Invitrogen); analytical window size 10.

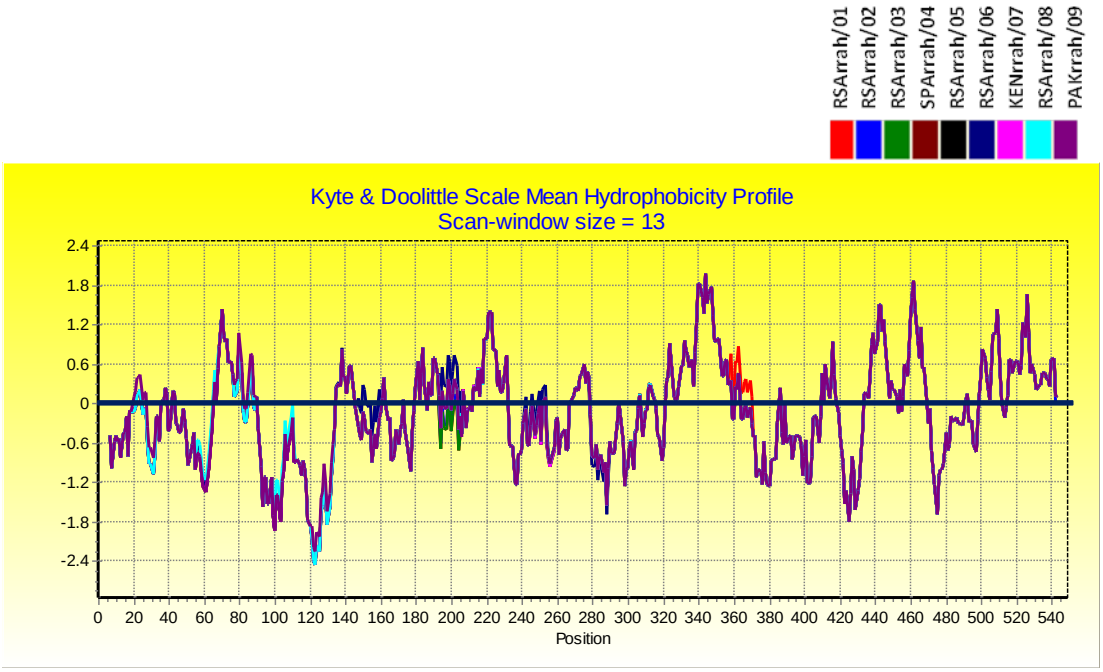


Figure 3.21: Hydrophobicity plot of the AHSV NS1 protein.
The plot was inferred using a Kyte and Doolittle Hydrophobicity Scale (Kweon *et al.*, 2003) accessed through BioEdit (Hall, 1999). Both hydrophobic and hydrophilic regions were predicted along NS1 amino acid sequence. Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0. Strains of different AHSV serotypes are represented by different coloured lines, as per key.

Seg-5 encodes NS1 (548 aa) from an ORF (nt 36 to 1682) that uses the same 'TGA' stop codon and is the same size in each of the strains analysed (Table 3.9). Hydrophobicity plots of NS1 shows conserved hydrophobic and hydrophilic regions (Figure 3.21). The NS1 aa sequences showed > 98% identity (RSArrah/06 and PAKrrah/09), but is identical in three strains (SPArrah/04, RSArrah/05 and KENrrah/07 - Table 3.10). Graphical representation of NS1 aa identity levels shows high levels of conservation within the protein, especially in its C-terminal region (Figure 3.20). The ratio of non-synonymous to synonymous substitutions per site (dN/dS = 0.067) indicates purifying selection of the

protein coding sequence, with low substitution rate of 3.3 x 10^{-2} nucleotides per site (Table 3.22).

Phylogenetic analyses using ML methods (Felsenstein, 1981) confirm the very close relationship between the Seg-5 / NS1 sequences of the AHSV reference strains (Figures 3.22 and 3.23). The topology of the nt and aa phylogenetic trees are very similar but are drawn at different scales to reflect the higher level of identity in the aa sequences. Trees were also built using NJ methods (Saitou and Nei, 1987) with similar results (data not shown).

Table 3.9: Genetic information for Seg-5/NS1 of the nine AHSV reference strains.

AHSV isolate name	Segment length (bp)	Open reading frame (bp)	G+C content (%)	Protein size (aa)	Predicted protein molecular mass (kDA)	Stop codon	5' Terminal sequence	3' Terminal sequence
RSArrah/01	1748	36-1682	41.7	548	63.3	TGA	GTTAAA…	...ACTTAC
RSArrah/02	1748	36-1682	41.6	548	63.3	TGA	GTTAAA…	...ACTTAC
RSArrah/03	1748	36-1682	41.8	548	63.3	TGA	GTTAAA…	...ACTTAC
SPArrah/04	1748	36-1682	41.8	548	63.2	TGA	GTTAAA…	...ACTTAC
RSArrah/05	1748	36-1682	41.8	548	63.2	TGA	GTTAAA…	...ACTTAC
RSArrah/06	1748	36-1682	41.8	548	63.2	TGA	GTTAAA…	...ACTTAC
KENrrah/07	1748	36-1682	41.7	548	63.2	TGA	GTTAAA…	...ACTTAC
RSArrah/08	1748	36-1682	41.9	548	63.1	TGA	GTTAAA…	...ACTTAC
PAKrrah/09	1748	36-1682	41.7	548	63.1	TGA	GTTAAA…	...ACTTAC

Table 3.10: Percentage nucleotide and amino acid sequence identities Seg-5 and NS1.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	99.5	99.8	99.5	99.5	98.2	99.5	98.7	98.5
RSArrah/02	99.4	ID	99.6	99.5	99.5	98.2	99.5	98.7	98.5
RSArrah/03	99.9	99.4	ID	99.6	99.6	98.4	99.6	98.9	98.7
SPArrah/04	98.8	99	98.9	ID	**100**	98.2	**100**	98.9	98.7
RSArrah/05	98.8	99	98.9	**100**	ID	98.2	**100**	98.9	98.7
RSArrah/06	96.2	96.5	96.3	96.4	96.4	ID	98.2	98.2	**98**
KENrrah/07	98.6	98.7	98.7	99.6	99.6	96.1	ID	98.9	98.7
RSArrah/08	97	96.9	97	96.9	96.9	95.9	96.7	ID	98.4
PAKrrah/09	96.2	96	96.2	96	96	**95.6**	95.9	95.9	ID

Identity levels in the nt (white area) and aa (blue shaded area) sequences of AHSV Seg-5 / NS1 were analysed for the nine reference strains. "ID" stands for identical sequences. Figures in bold highlight minimum percentage identity between strains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

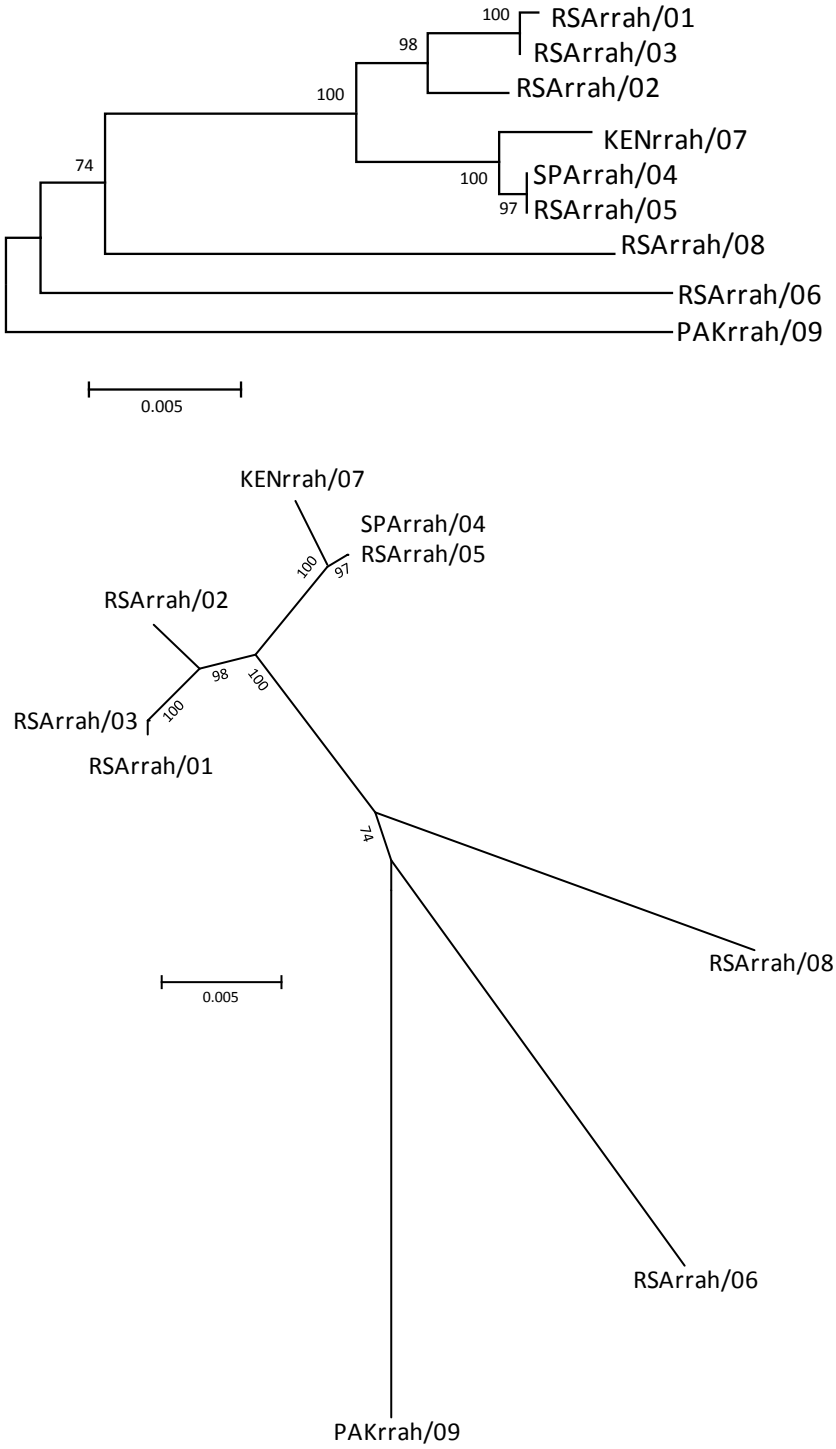


Figure 3.22: Phylogenetic analysis of AHSV Seg-5 nucleotide sequences.
Phylogenetic analyses of nt sequences of Seg-5 from the AHSV reference strains using a ML method based on the Tamura-Nei model (TN93) (Tamura & Nei, 1993) with a discrete Gamma distribution (rectangular (top) and radial trees(bottom)). The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

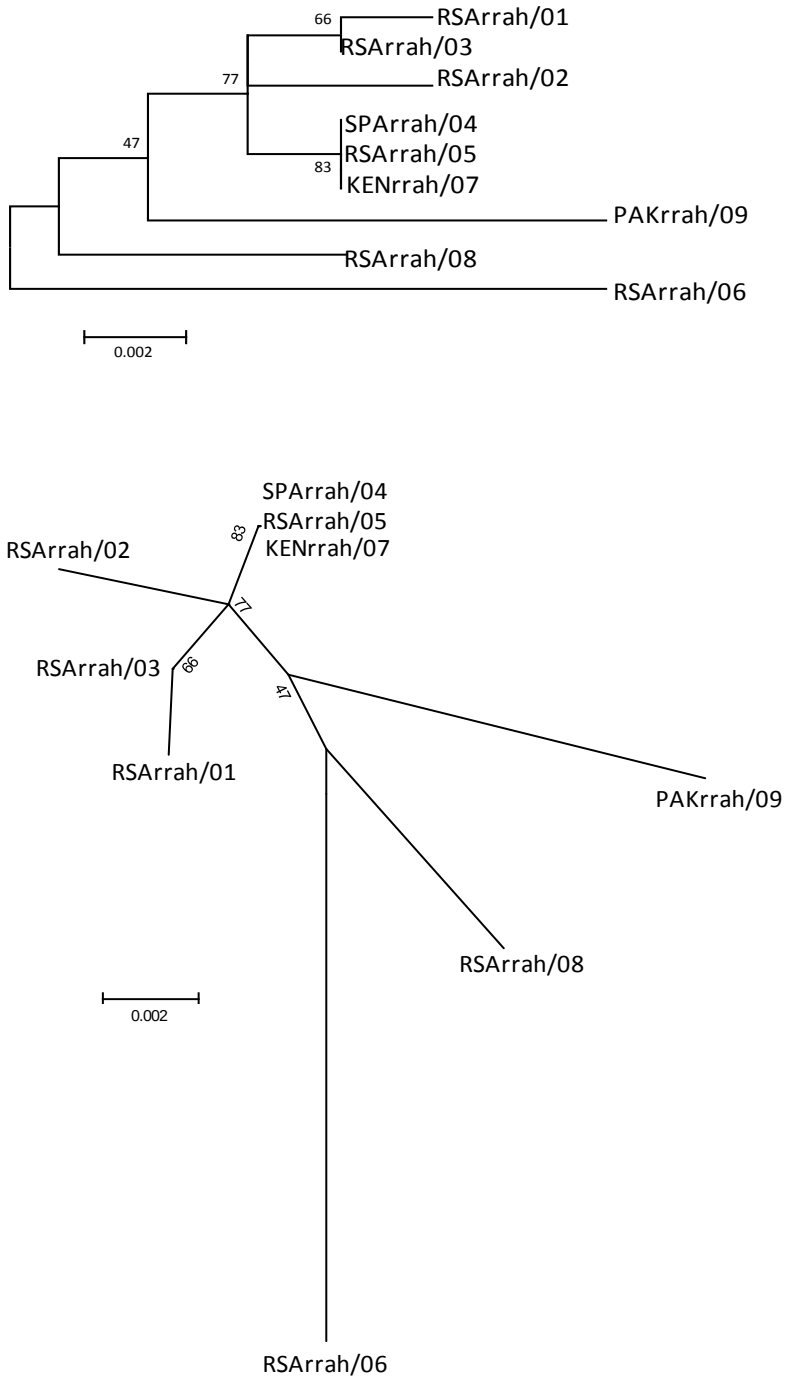


Figure 3.23 Phylogenetic analysis of AHSV NS1 amino acid sequence.
Phylogenetic analysis of aa sequences of NS1 from the nine AHSV reference strains are presented as rectangular (top) and radial (bottom) trees. The analysis uses a ML method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992). The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

3.2.3.6: Genome segment 6

Seg-6 of the AHSV reference strains is 1564 to 1566 bp long, with a G+C content of 43 to 43.7% (Table 3.11). In each case the 5' NCR is 19 nt and contains two variable regions (Figure 3.24); one which is within the 5' terminal hexanucleotide sequence (Table 3.11). Overall identity within this NCR is ≥ 84.2% for the strains investigated. The consensus sequence for the 5' terminal hexanucleotide can be expressed as 5'-GTT$^{A}/_{T}$AT...3' (Table 3.11).

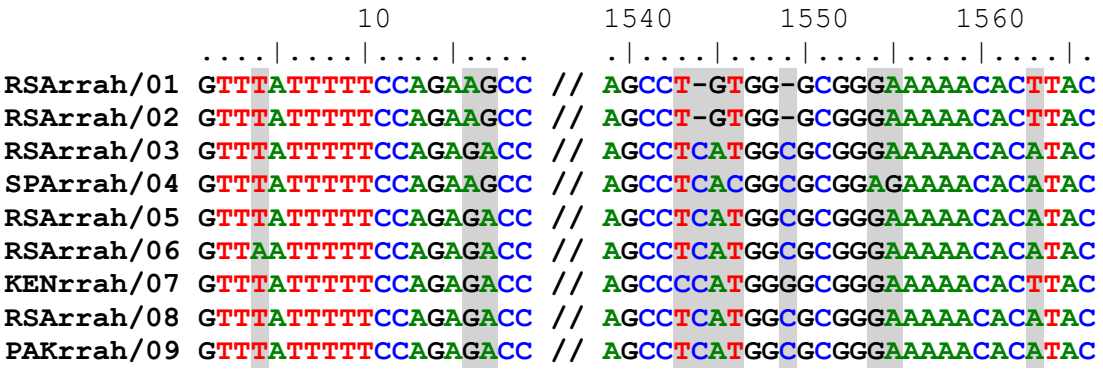


Figure 3.24: Alignment of the 5' and 3' non-coding regions (NCR) of AHSV Seg-6. The open reading frame (ORF) has been removed and replaced with "//". Variable regions between the strains investigated are highlighted in grey. Figure prepared in BioEdit (Hall, 1999).

Differences in the total length of Seg-6 arise from a two-nucleotide deletion within the 3' NCR regions of RSArrah/01 and RSArrah/02 in comparison to the remaining reference strains. Consequently, the 3' NCR region also oscillates between 27 and 29 nt with overall identity of >80% for the strains investigated (Figure 3.11). Variability in this NCR is also reflected in the 3' terminal hexanucleotide which can be represented as 3'...AC$^{A}/_{T}$TAC-5' (Table 3.11). Seg-6 shows a considerable level of variation (> 70.3% identity) between the isolates / serotypes studied (Table 3.12), identifying this as one of the more variable segments of the AHSV genome. Despite this variability, two reference

strains (RSArrah/05 and RSArrah/08) contain and identical Seg-6 (Table 3.12), providing evidence for a common ancestry and reassortment events during the evolution of these strains. A plot of variation within the full sequence of Seg-6 from the reference strains shows alternate peaks of increased identity and dips of variability (Figure 3.25).

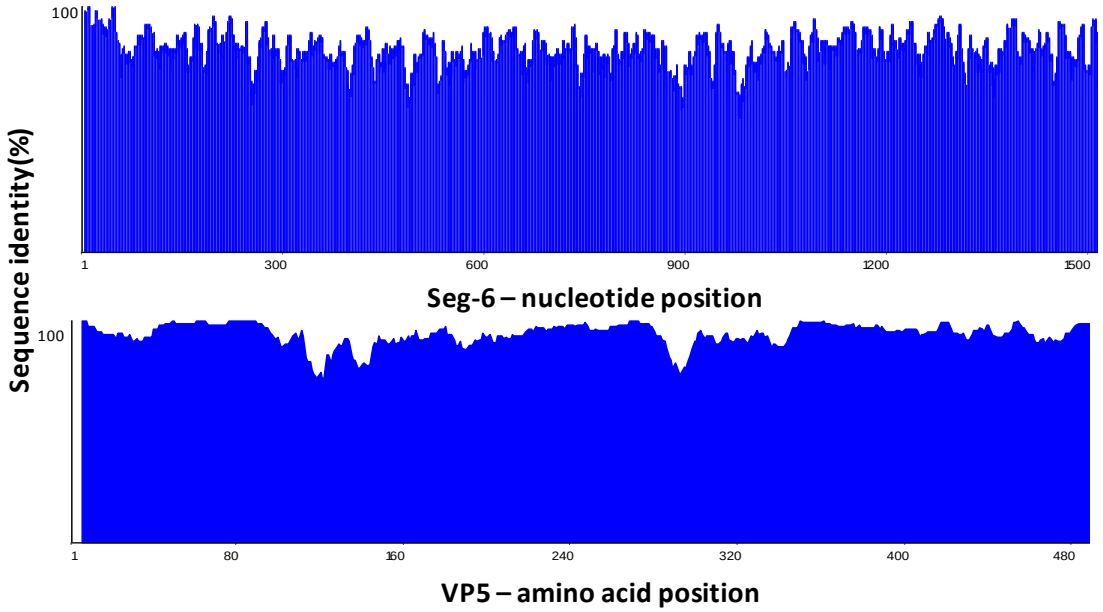


Figure 3.25: Graphical representations of nucleotide and amino acid sequence identities of Seg-6/VP5.
Peaks of increased identity are alternated by more variable regions in Seg-6 /VP5 of the nine reference strains. Alignments and plots were constructed using VectorNTI software (Invitrogen); analytical window size 10.

AHSV Seg-6 encodes outer capsid protein VP5, from a single large ORF positioned between base pairs 20-1537 for all nine reference strains. Two different stop codons are used: TAG in RSArrah/01 and RSArrah/02, while TGA in the remaining reference strains (Table 3.11). The ORF codes for 505 aa (56.6 – 57 kDa) (Table 3.11) which is predicted to have strong hydrophilic regions alternated by hydrophobic ones (Figure 3.26). The overall aa identity level for these strains of AHSV VP5 is > 76.8% (Table 3.12),

identifying it as the third most 'variable' protein of AHSV. However, this variability is not evenly distributed throughout the protein but appear to concentrate at a number of specific regions which are alternated by relatively more conserved sections (Figure 3.25). Despite the relatively high variability within VP5, the ratio of non-synonymous (d_N) to synonymous (d_S) substitutions per site of the amino acid sequence indicates of purifying selection (d_N/d_S = 0.064). The predicted nucleotide substitution rate of 0.34 nucleotide substitutions per site within the open reading frame is relatively high in comparison with sequences of the more conserved genome segments of the analysed viruses (Table 3.22).

Phylogenetic analysis of the nt and aa sequences of Seg-6 / VP5 from the AHSV reference strains generated trees with very similar topology although with different 'scales' (Figures 3.27 and 3.28). Overall the different reference strains grouped into clusters similar to those described above for Seg-2 / VP2, with the exception of RSArrah/03 which grouped with RSArrah/06 and PAKrrah/09 rather than with KENrrah/07 (which consequently formed a group by itself). Two isolates, RSArrah/05 and RSArrah/08, have the same nt and amino acid sequence for Seg-6, indicating that in Seg-6 / VP5 does not determine serotype for these viruses. Phylogenetic trees presented in these studies were drawn using a ML method (Felsenstein, 1981). NJ phylogenetic trees were also constructed for comparison purposes and showed similar topology (Saitou & Nei, 1987) (data not shown).

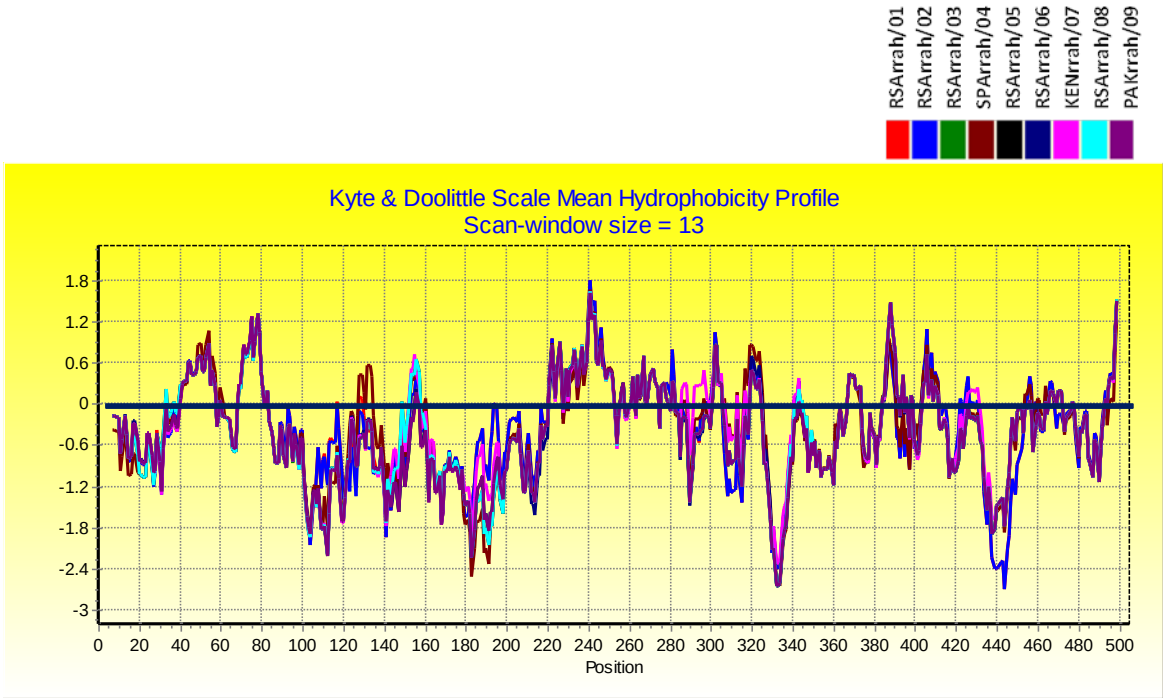


Figure 3.26: Hydrophobicity plot of the AHSV VP5 proteins.

The plot was drawn using a Kyte and Doolittle Hydrophobicity Scale (Kyte and Doolittle, 1982) accessed through BioEdit (Hall, 1999). Both hydrophobic and hydrophilic regions were predicted along the VP5 aa sequence but clear differences in hydrophobicity profiles of individual isolates are present. Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0. Strains of different AHSV serotypes are represented by different coloured lines, as per key.

Table 3.11: Genetic information for Seg-6/VP5 of AHSV reference strains.

AHSV isolate name	Segment length (bp)	Open reading frame (bp)	G+C content (%)	Protein size (aa)	Predicted protein molecular mass (kDA)	Stop codon	5' Terminal sequence	3' Terminal sequence
RSArrah/01	1564	20-1537	43	505	56.6	TAG	GTTTAT…	…ACTTAC
RSArrah/02	1564	20-1537	43.5	505	56.6	TAG	GTTTAT…	…ACTTAC
RSArrah/03	1566	20-1537	43.1	505	56.9	TGA	GTTTAT…	…ACATAC
SPArrah/04	1566	20-1537	44.3	505	56.8	TGA	GTTTAT…	…ACATAC
RSArrah/05	1566	20-1537	43.5	505	56.9	TGA	GTTTAT…	…ACATAC
RSArrah/06	1566	20-1537	43.3	505	57	TGA	GTTAAT…	…ACATAC
KENrrah/07	1566	20-1537	43.7	505	56.8	TGA	GTTTAT…	…ACTTAC
RSArrah/08	1566	20-1537	43.5	505	56.9	TGA	GTTTAT…	…ACATAC
PAKrrah/09	1566	20-1537	43.7	505	56.9	TGA	GTTTAT…	…ACATAC

Differences in nucleotide sequences are highlighted in dark blue.

Table 3.12: Percentage identities between Seg-6 nucleotide and VP5 amino acid sequences.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	98.6	78.8	77	78.2	78.8	78.2	78.2	78.8
RSArrah/02	85.5	ID	79.2	**76.8**	78.6	79.2	78	78.6	79.4
RSArrah/03	71.9	71.5	ID	84.6	96.6	98	90.1	96.6	98.4
SPArrah/04	71.9	71.1	75.2	ID	84.2	84.6	82	84.2	84.2
RSArrah/05	72.9	71.9	83.5	76.6	ID	95.8	88.9	**100**	96.2
RSArrah/06	71.5	72.1	96.6	75.2	83.1	ID	89.3	95.8	97.6
KENrrah/07	71.1	72	78	73.4	78	78	ID	88.9	89.7
RSArrah/08	72.9	71.9	83.5	76.6	**100**	83.1	78	ID	96.2
PAKrrah/09	**70.3**	71.7	96.2	75	83.3	95.4	77.3	83.3	ID

Identity levels in the nt (white area) and aa (blue shaded area) sequences of AHSV Seg-6 / VP5 were analysed for the nine reference strains. "ID" stands for identical sequences. Figures in bold highlight minimum percentage identity between strains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011)

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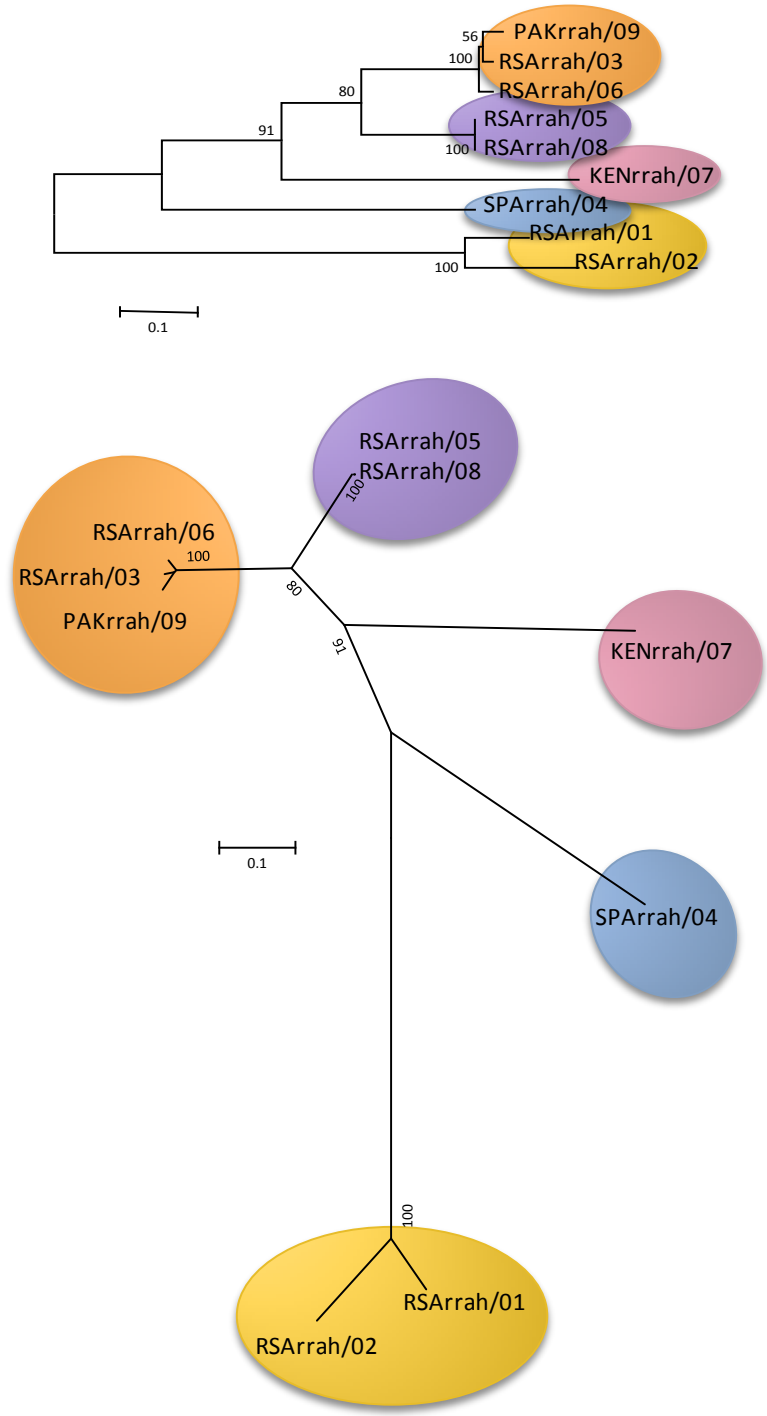


Figure 3.27: Phylogenetic analysis of Seg-6 nucleotide sequences.

Phylogenetic analyses of nt sequences of Seg-6 from the AHSV reference strains using a ML method based on the Tamura-Nei model (TN93) (Tamura & Nei, 1993) with a discrete Gamma distribution (rectangular (top) and radial trees(bottom)). The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

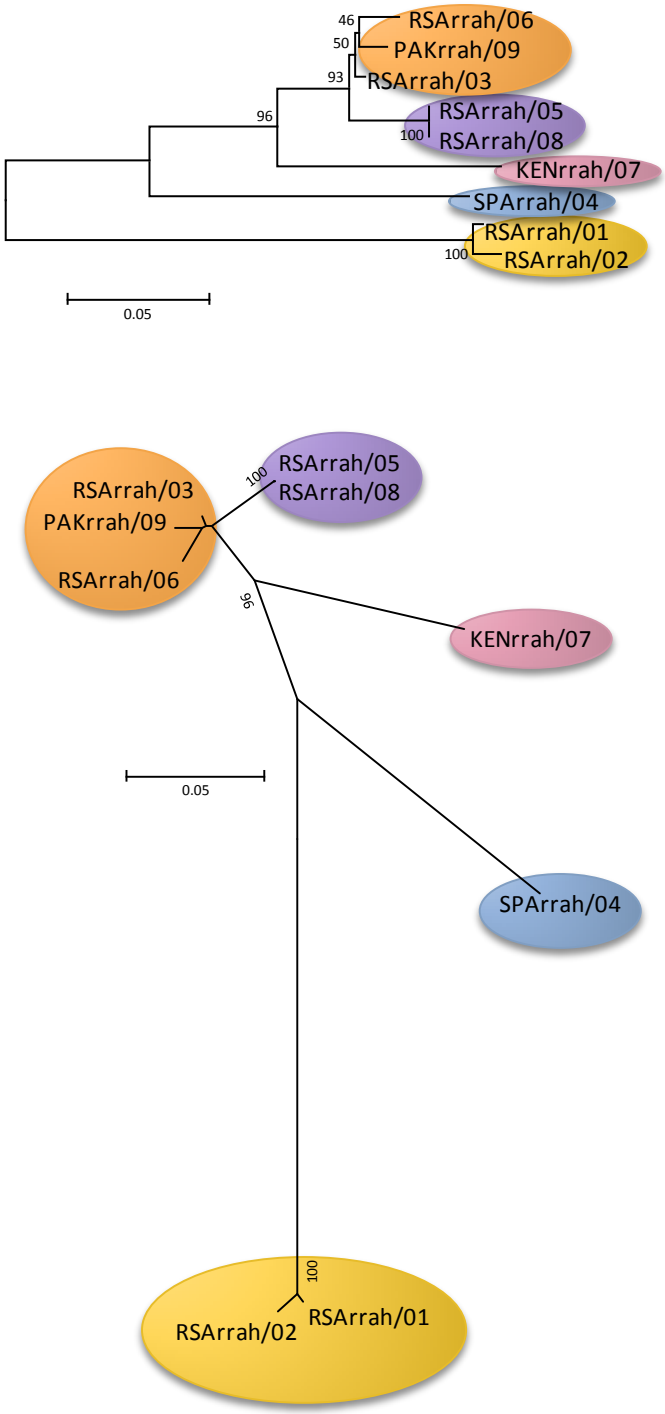


Figure 3.28: Phylogenetic analysis of VP5 amino acid sequences.

Phylogenetic analysis of aa sequences of VP5 from the nine AHSV reference strains are presented as rectangular (top) and radial (bottom) trees. The analysis uses a ML method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992) with a discrete Gamma distribution. The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

3.2.3.7: Genome segment 7

AHSV Seg-7 has a C + G content of 47.7% to 48% (Table 3.13). It is 1169 nt in length in most of the reference strains investigated but a 6-nucleotide deletion at position 541-546 reduces Seg-7 of RSArrah/08 to 1163 nt (Table 3.13). The 5' and 3' NCRs are 17 and 42 nt long respectively, and are identical in all of the reference strains (Table 3.13). The 5' and 3' terminal hexanucleotide sequences are therefore also conserved as 5'-GTTAAA…ACTTAC-3' (Table 3.13).

Seg-7 is highly conserved and the strains investigated share > 96.2% nucleotide identity. Two isolates, RSArrah/01 and RSArrah/06, have the same version of this genome segment (Table 3.14), providing further evidence of a common ancestry and reassortment events during the evolution of these strains. Nucleotide differences between different strains are distributed throughout the length of this genome segment, although regions of total conservations are also evident (Figure 3.29).

AHSV Seg-7 codes for two proteins: VP6 (the viral helicase) and NS4 (a non-structural protein) from two independent open reading frames (Belhouchet *et al.*, 2011; O'Hara *et al.*, 1998; Ratinier *et al.*, 2011). The VP6 ORF is located between base pairs 18 to 1127 or, between base pairs 18 to 1121 (for RSArrah/08), and is translated into a 369 or 367 aa protein with molecular mass of 38.1 to 38.4 kDa, respectively (Table 3.13). The same 'TAG' stop codon, is used to terminate translation of VP6 in all of the strains analysed (Table 3.13).

The second, smaller and downstream but overlapping ORF of Seg-7, codes for NS4 in the '+1' reading frame, starting from 193 bp, with a TAG stop codon at position 655 bp (649 bp in RSArrah/08). However, the consensus sequence of RSArrah/02, RSArrah/05 and PAKrrah/09 indicate early termination of the protein, as a result of a single nucleotide

substitution, at positions of 579, 600 and 534 bp respectively (Table 3.13). Careful examination of the sequencing chromatograms in these locations, showed double peaks suggesting co-expression of both shorter and longer forms of the NS4 protein in RSArrah/02, RSArrah/05 and PAKrrah/09.

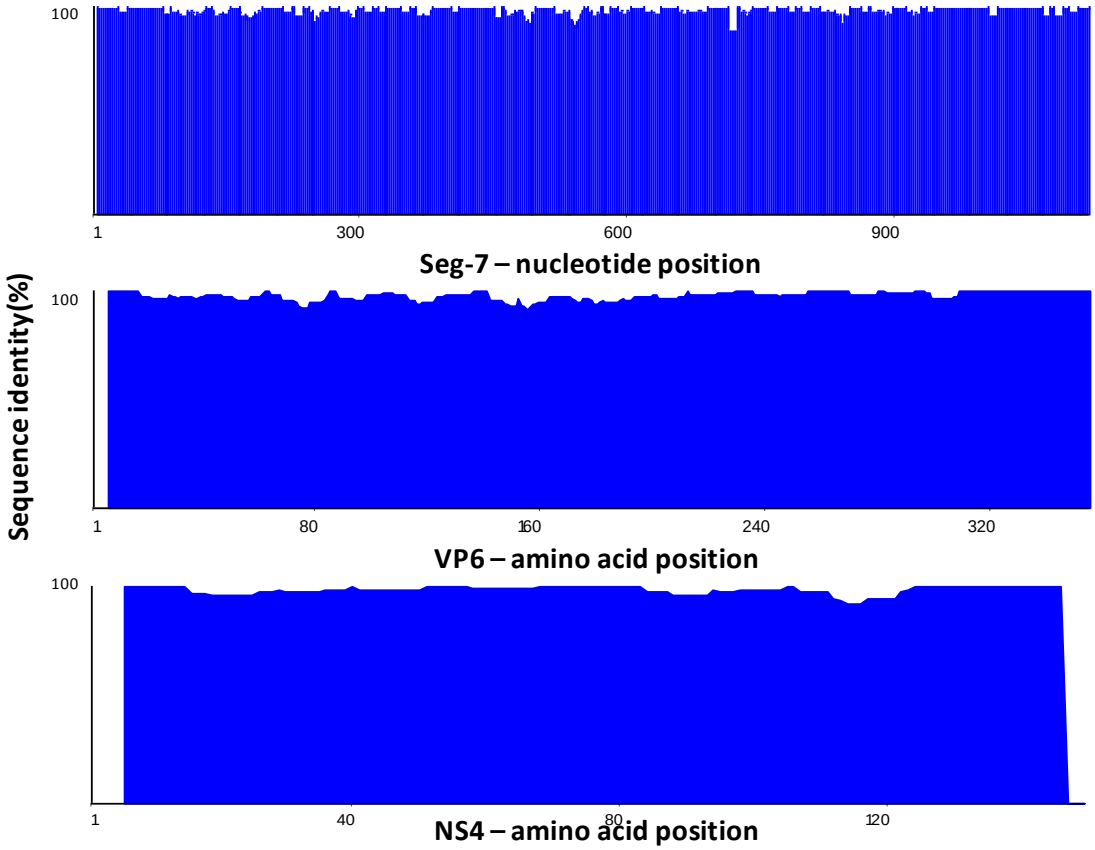


Figure 3.29: Graphical representation of nucleotide and amino acid sequence identities of Seg-7/VP6/NS4. Some variability was detected between nt sequences of Seg-7, which translates as a low level of variability in the amino acid sequences, mainly of VP4. In both, VP6 and NS4, sequences variability is concentrated in the central parts of the proteins. Alignments and plots were constructed using VectorNTI software (Invitrogen); analytical window size 10.

The "long" version of NS4 ORF, for which further analysis of NS4 apply, translates as 154 or 152 (in RSArrah/08) aa protein with 18 to 18.2 kDa molecular mass (Table 3.13).

As predicted from the hydrophobicity plots (Figure 3.30 and 3.31) VP6 is mainly hydrophobic, while NS4 contains both hydrophilic and hydrophobic regions. Amino acid sequences at both ends are highly conserved in both of the proteins encoded by Seg-7, while some variation was observed in the central regions of both proteins (Figure 3.29).

Overall, the nt identity level of Seg-7 shows a lower minimum value than the aa sequence for either of the proteins VP6 (> 93.8%) and NS4 (95.7%) (Tables 3.14 and 3.15). This indicates the occurrence of both synonymous and non-synonymous substitutions during the evolution of Seg-7. Of the AHSV genome segments analysed, similar observations has been made only in Seg-2 (Section 3.2.3.2). The estimated overall non-synonymous (d_N) to synonymous (d_S) substitutions per site for NS4 (d_N/d_S = 0.289) is of similar value to Seg-2, while for VP6 (d_N/d_S = 0.883) it is the highest of any calculated for an AHSV proteins; although still indicating purifying selection for the isolates investigated (Table 3.22). The nucleotide substitution rate for the VP6 ORF is of 2.1 x 10^{-2} substitutions per site (comparable to the substitution rate for the VP5 ORF of Seg-6 of 3.4 x 10^{-1} substitutions per site) while the same calculations for NS4 produced a lower value of 2.8 x 10^{-2} nucleotide substitutions per site indicating that this region of the genome segment is more highly conserved (Table 3.22). This is perhaps unsurprising as it contains two overlapping ORFs.

Phylogenetic comparisons of Seg-7 nt and aa sequences, for both proteins encoded by this segment, demonstrate close relatedness between the AHSV strains (Figure 3.32, 3.33 and 3.34). The phylogenetic grouping of each of the sequence data sets analysed show similar topology where (apart from RSArrah/05 and RSArrah/08) respective isolates group independently of serotype (as observed in phylogenetic analysis of Seg-2 and Seg-6). Two isolates, RSArrah/01 and RSArrah/06 share the same nucleotide, NS4 and VP6

sequences suggesting common ancestry and reassortment in the evolution of these strains. Phylogenetic analyses were carried out using ML methods (Felsenstein, 1981) but, for comparative purposes, NJ trees were also produced showing similar topologies (Saitou & Nei, 1987) (data not shown).

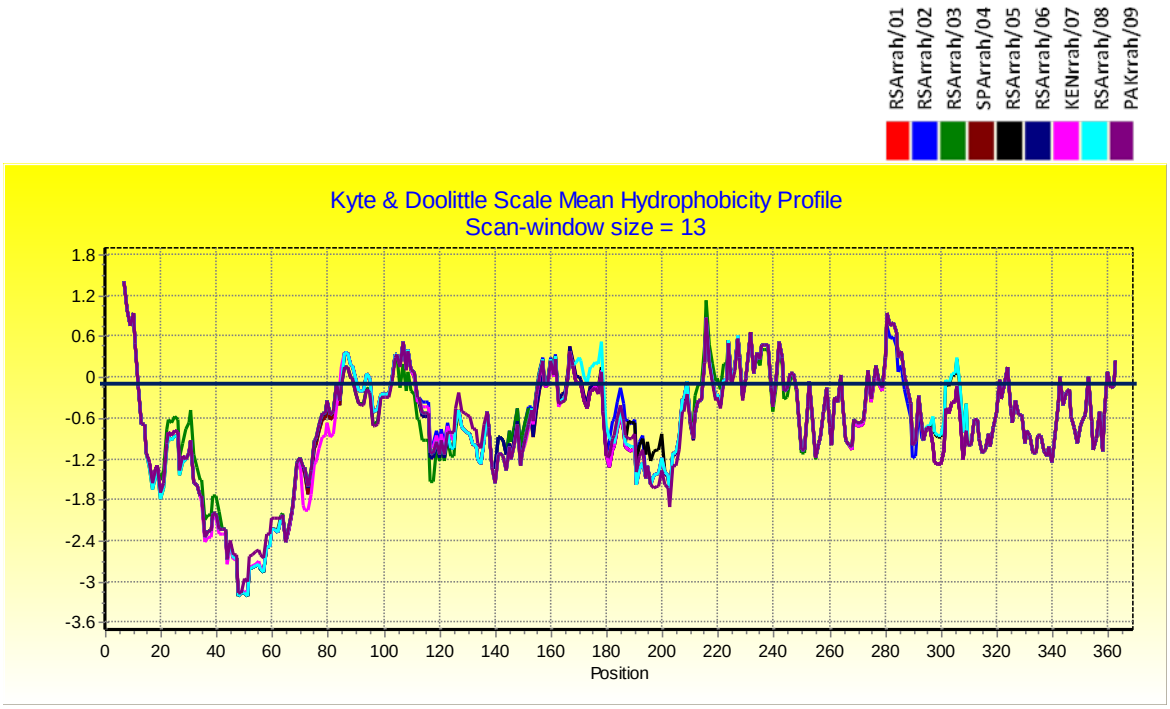


Figure 3.30: Hydrophobicity plot of the VP6 protein.
The hydrophobicity of VP6 was plotted using a Kyte and Doolittle Hydrophobicity Scale (Kyte and Doolittle, 1982) accessed through BioEdit (Hall, 1999)(Hall, 1999). VP6 was identified as mainly hydrophilic protein. Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0. Strains of different AHSV serotypes are represented by different coloured lines, as per key.

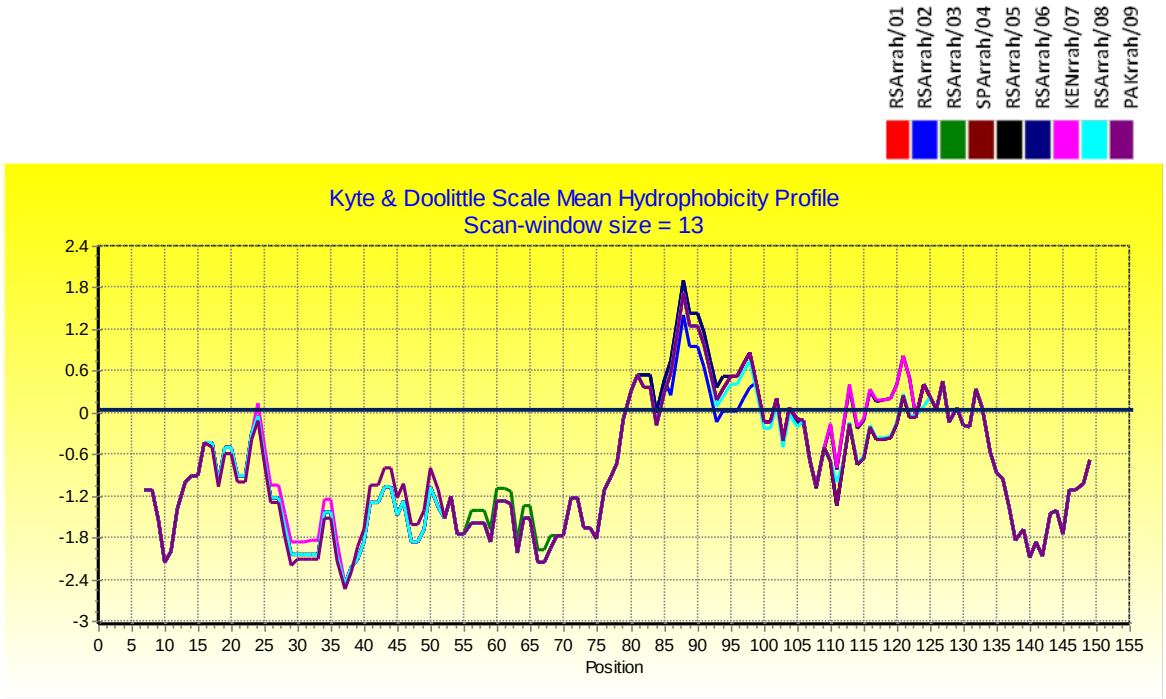


Figure 3.31: Hydrophobicity plot of the NS4 protein. The plot was inferred using Kyte and Doolittle Hydrophobicity Scale (Kyte and Doolittle, 1982) accessed through BioEdit (Hall, 1999). NS4 was inferred as mainly hydrophilic with a pronounced hydrophobic region but clear differences in hydrophobicity profiles of individual isolates are present throughout the length of the protein. Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0. Strains of different AHSV serotypes are represented by different coloured lines, as per key.

Table 3.13: Genetic information for Seg-7, VP6 and NS4 of the AHSV reference strains.

AHSV isolate name	Segment length (bp)	G+C content (%)	VP6 open reading frame (bp)	VP6 Protein size (aa)	VP6 Predicted protein molecular mass (kDA)	VP6 Stop codon	NS4 open reading frame (bp)	NS4 Protein size (aa)	NS4 Predicted protein molecular mass (kDA)	NS4 Stop codon	5' Terminal sequence	3' Terminal sequence
RSArrah/01	1169	47.8	18-1127	369	38.3	TAG	193-657	154	18.1	TAG	GTTAAA…	…ACTTAC
RSArrah/02	1169	47.9	18-1127	369	38.4	TAG	193-657	154	18.1	TAG	GTTAAA…	…ACTTAC
RSArrah/02	1169	47.9	18-1127	369	38.4	TAG	**193-579**	**128**	15.2	TAG	GTTAAA…	…ACTTAC
RSArrah/03	1169	48	18-1127	369	38.4	TAG	193-657	154	18.1	TAG	GTTAAA…	…ACTTAC
SPArrah/04	1169	47.7	18-1127	369	38.4	TAG	193-657	154	18.2	TAG	GTTAAA…	…ACTTAC
RSArrah/05	1169	47.5	18-1127	369	38.4	TAG	193-657	154	18.2	TAG	GTTAAA…	…ACTTAC
RSArrah/05	1169	47.5	18-1127	369	38.4	TAG	**193-600**	**135**	15.9	**TAA**	GTTAAA…	…ACTTAC
RSArrah/06	1169	47.8	18-1127	369	38.3	TAG	193-657	154	18.1	TAG	GTTAAA…	…ACTTAC
KENrrah/07	1169	47.8	18-1127	369	38.4	TAG	193-657	154	18.2	TAG	GTTAAA…	…ACTTAC
RSArrah/08	**1163**	47.7	**18-1121**	**367**	38.1	TAG	**193-651**	**152**	18	TAG	GTTAAA…	…ACTTAC
PAKrrah/09	1169	48	18-1127	369	38.3	TAG	193-657	154	18.2	TAG	GTTAAA…	…ACTTAC
PAKrrah/09	1169	48	18-1127	369	38.3	TAG	**193-534**	**113**	13.7	TAG	GTTAAA…	…ACTTAC

Differences in length and ORFs between strains are in bold blue face.

Table 3.14: Percentage identities between Seg-7 nucleotide and VP6 amino acid sequences.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	96.5	95.1	97.8	96.7	**100**	97.3	96.7	94.3
RSArrah/02	97.9	ID	95.7	98.1	96.5	96.5	96.5	96.5	94.9
RSArrah/03	97.2	97.9	ID	96.2	95.1	95.1	94.3	95.1	**93.8**
SPArrah/04	98.7	99	98	ID	98.4	97.8	97.8	98.4	95.9
RSArrah/05	98.4	98.5	97.8	99.4	ID	96.7	96.2	99.5	95.4
RSArrah/06	**100**	97.9	97.2	98.7	98.4	ID	97.3	96.7	94.3
KENrrah/07	98.7	97.9	96.7	98.6	98.1	98.7	ID	96.2	94.3
RSArrah/08	98.2	98.4	97.6	99.2	99.7	98.2	97.9	ID	95.4
PAKrrah/09	96.3	96.7	**96.2**	97.2	97	96.3	**96.2**	96.8	ID

Identity levels in the nt (white area) and aa (blue shaded area) sequences of AHSV Seg-7 / VP6 were analysed for the nine reference strains. "ID" stands for identical sequences. Figures in bold highlight minimum percentage identity between strains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

Table 3.15: Percentage identities between NS4 nucleotide and NS4 amino acid sequences.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	97.6	97.4	98.3	98.1	**100**	98.1	97.8	95.9
RSArrah/02	97.4	ID	98.1	98.9	98.3	97.6	97.8	98	96.6
RSArrah/03	98	98	ID	98.3	98.1	97.4	96.8	97.8	96.8
SPArrah/04	97.4	98.7	98	ID	99.4	98.3	98.5	99.1	97.2
RSArrah/05	98	98	98.7	99.3	ID	98.1	97.8	99.8	97.4
RSArrah/06	**100**	97.4	98	97.4	98	ID	98.1	97.8	95.9
KENrrah/07	97.4	96.7	96.7	98	98	97.4	ID	97.6	**95.7**
RSArrah/08	97.3	97.3	98	98.7	99.3	97.3	97.3	ID	97.2
PAKrrah/09	**96**	**96**	96.7	97.4	98	**96**	**96**	97.3	ID

Identity levels in the nt (white area) and aa (blue shaded area) sequences of the AHSV-NS4 ORF and NS4 protein were analysed for the nine reference strains. "ID" stands for identical sequences. Figures in bold highlight minimum percentage identity between strains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

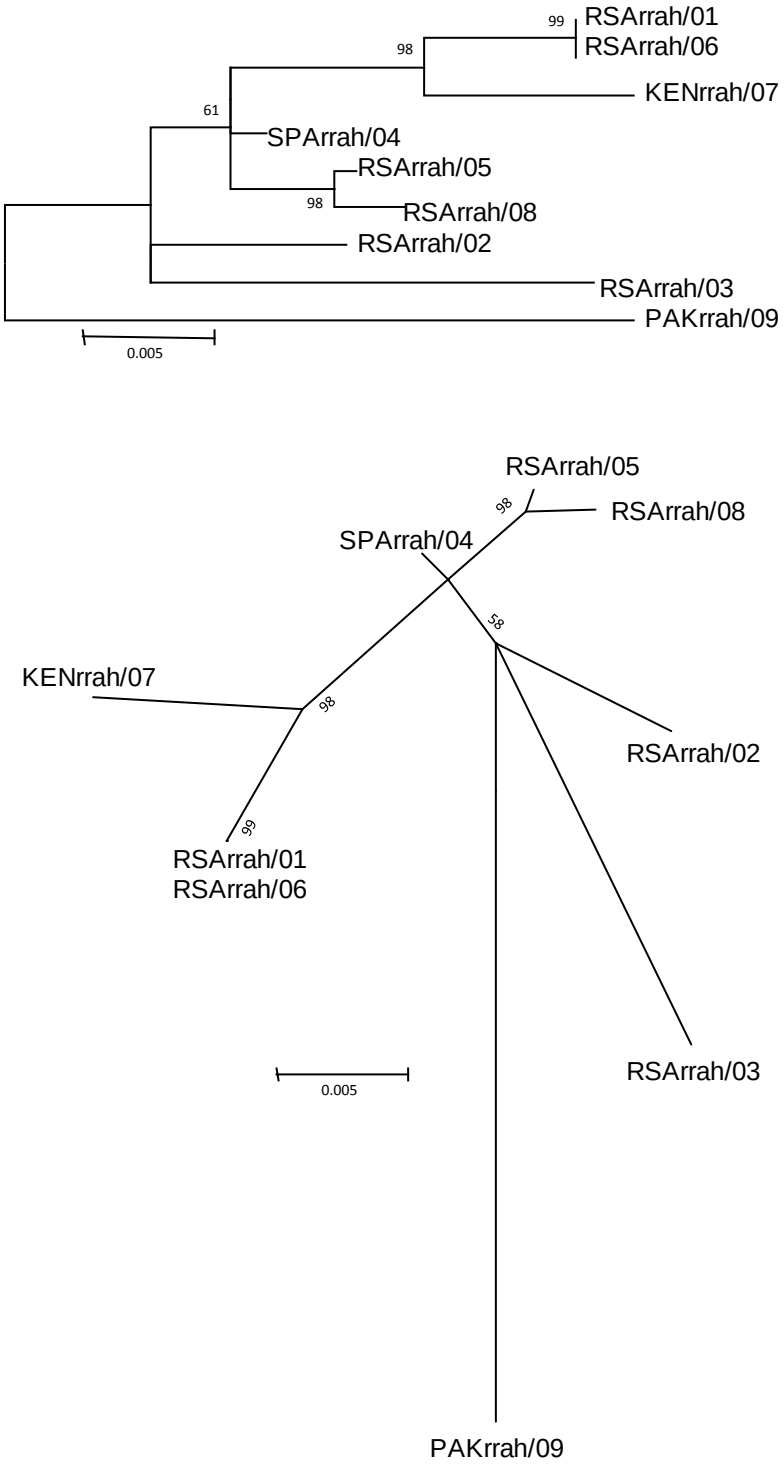


Figure 3.32: Phylogenetic analysis of AHSV Seg-7 nucleotide sequences.
Phylogenetic analyses of nt sequences of Seg-7 from the AHSV reference strains using a ML method based on the Hasegawa-Kishino-Yano model (HKY) (Hasegawa *et al.*, 1985) with a discrete Gamma distribution. (rectangular (top) and radial trees(bottom)). The trees are drawn with branch lengths measured by the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

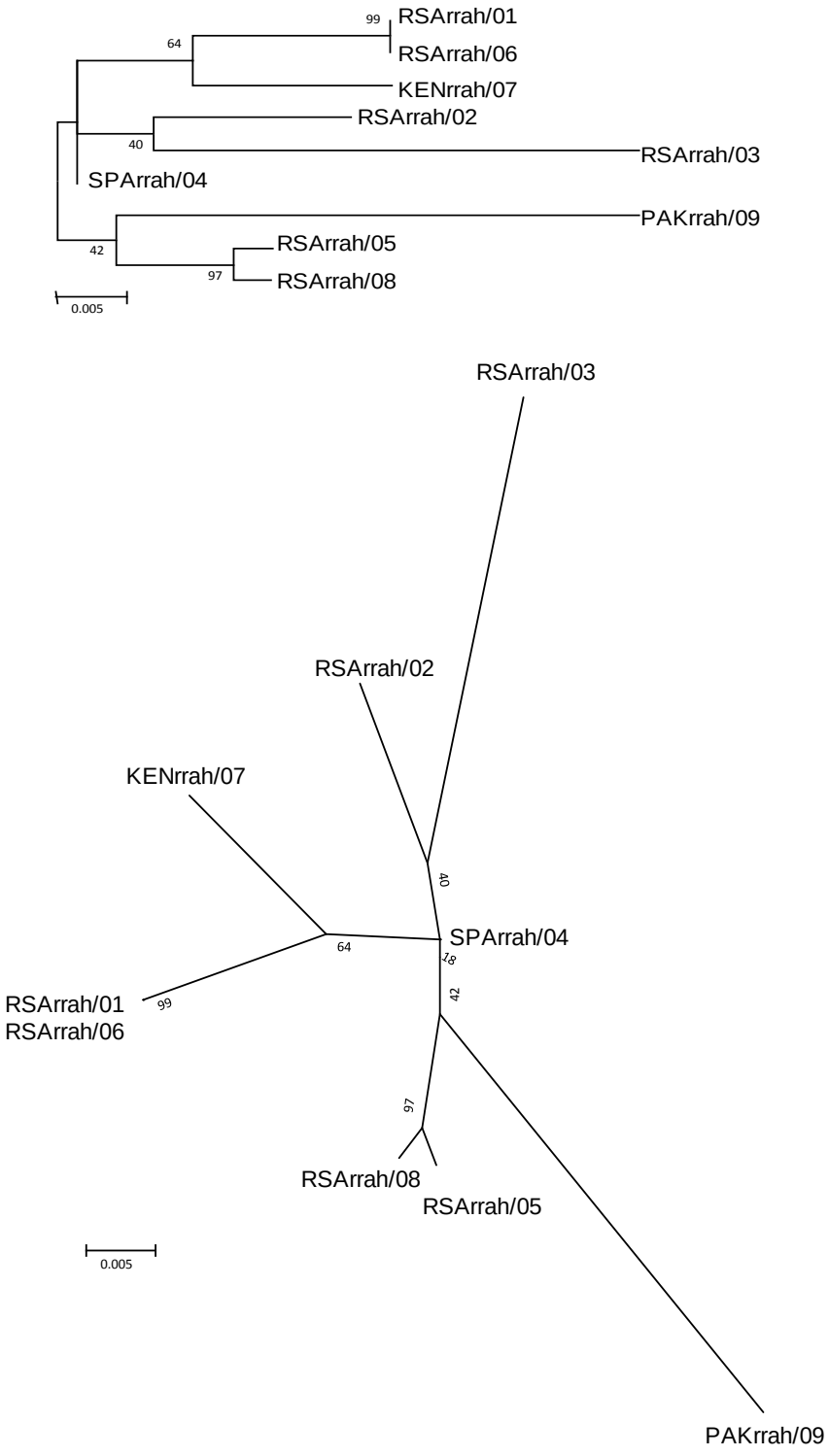


Figure 3.33: Phylogenetic analysis of AHSV VP6 amino acid sequences.
Phylogenetic analysis of aa sequences of VP6 from the nine AHSV reference strains are presented as rectangular (top) and radial (bottom) trees. The analysis uses a ML method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992) with a discrete Gamma distribution. The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

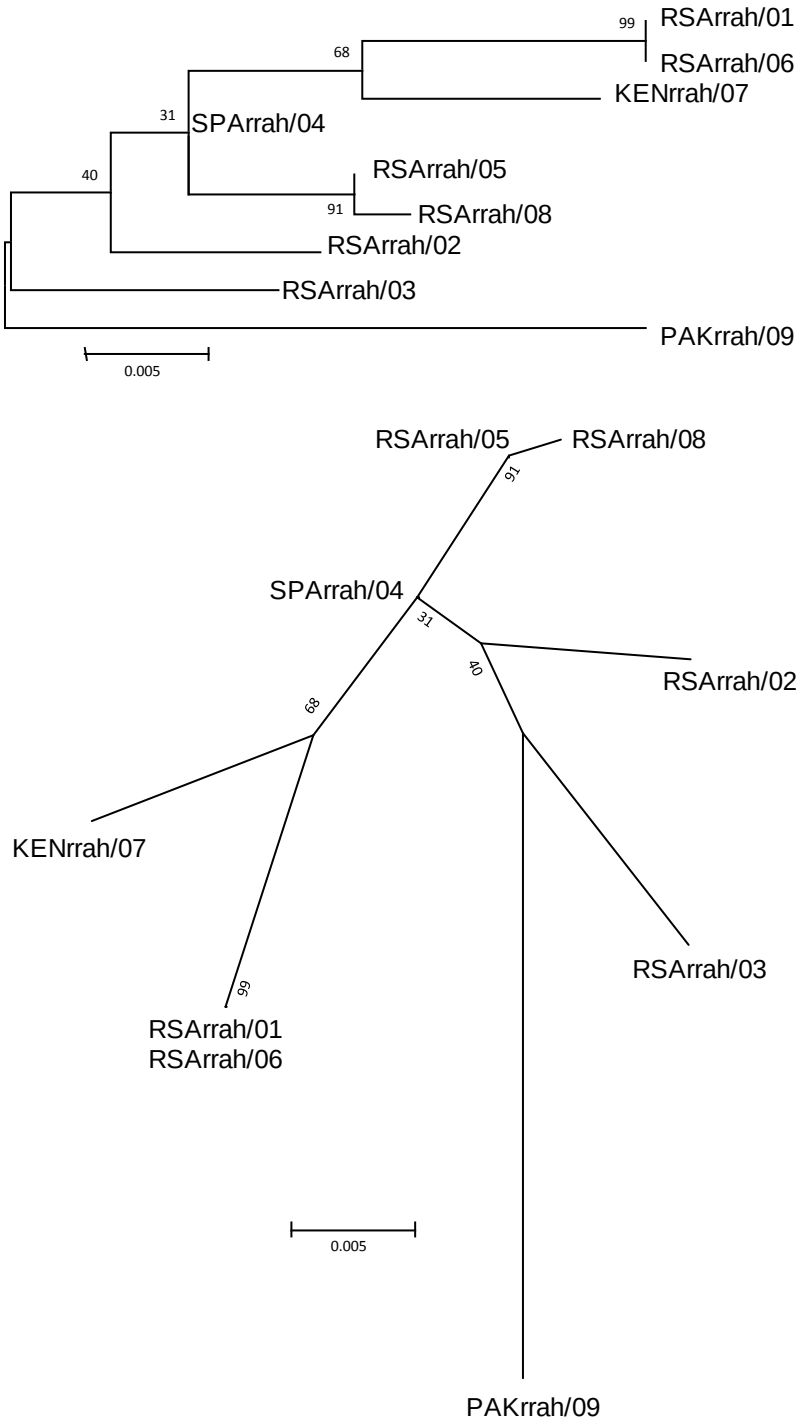


Figure 3.34: Phylogenetic analysis of AHSV NS4 amino acid sequences.
Phylogenetic analysis of aa sequences of NS4 from the nine AHSV reference strains are presented as rectangular (top) and radial (bottom) trees. The analysis used a ML method based on the Whelan and Goldman + Freq. model (WAG+F) (Whelan & Goldman, 2001) with a discrete Gamma distribution. The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

3.2.3.8: Genome segment 8

AHSV Seg-8 is 1162 nt in length for all of the nine reference strains investigated with a G+C content between 46-46.5 (Table 3.16). The 5' NCR is 17 bp and is conserved apart from two independent nucleotide substitutions within the 5' terminal hexanucleotide (Table 3.16). The 3' NCR is significantly longer (100 bp) and shows >97.3 % identity for the viruses analysed. The consensus sequences for the 5' and 3' terminal hexanucleotides of Seg-8 can be presented as 5'-GTT$^{A}/_{T}$AT... AC$^{C}/_{T}$TAC 3' (Table 3.16). The nt sequences of Seg-8 from the AHSV reference strains show ≥89.1% identity (Table 3.17). This value was achieved due to two reference strains (RSArrah/02 and RSArrah/05) grouping closely together but at a relative distance from the remaining isolates (Table 3.17, Figure 3.37). The pooled, sequence identity plot shows a high level of conservation at both ends of the analysed sequences, in comparison to a moderately variable central part of the genome segment (Figure 3.35).

Seg-8 codes for the outer core protein VP7, from an ORF located at nucleotides 18 to 1067 for all the strains analysed in this study, using the same 'TAG' stop codon (Table 3.16). The ORF is translated into a 347 aa protein with clear hydrophilic and hydrophobic regions (Figure 3.36) and in each case a molecular weight of 37.8 KDa (Table 3.16).

A conserved 'RGD' motif, thought (potentially) to be responsible for VP7 biding activity to *Culicoides* cells by BTV cores (Tan *et al.*, 2001), has been identified in the aa sequence alignments of the nine reference strains of AHSV, at positions 178-180 bp. High overall identity levels of >98% were detected between the aa sequences of VP7 from the different reference strains (Table 3.17). This indicates a high level of conservation in this protein, possibly due to its role in maintaining the structure of the virion, and correlates with its role as the major serogroup specific antigen. However, the overall percentage

identity of VP7 is reduced by RSArrah/02 and RSArrah/05, which group distantly from the remaining isolates (Figure 3.38). The percentage of aa identity within the two separate VP7 clusters > 99.4% (Table 3.17). Three groups of the strains investigated (RSArrah/01 and RSArra/06; RSArrah/03 and PAKrrah/09; SPArrah/04, KENrrah/07 and RSArrah/08) were shown to express identical versions of VP7. The first two of these groups also contained identical versions of Seg-8 (Table 3.17).

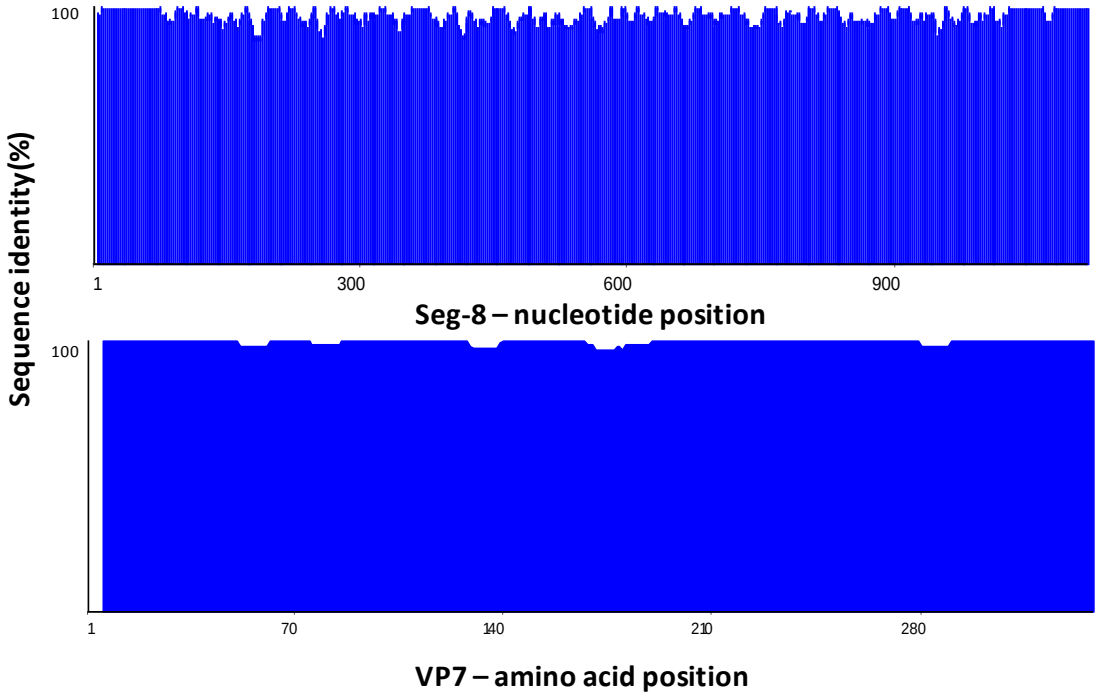


Figure 3.35: Graphical representation of AHSV Seg-8 / VP7 nucleotide and amino acid sequence identities.

Moderate variability in the nucleotide sequence of Seg-8 is translated into scattered variations at the aa level in VP7. Alignments and plots were constructed using VectorNTI software (Invitrogen); analytical window size 10.

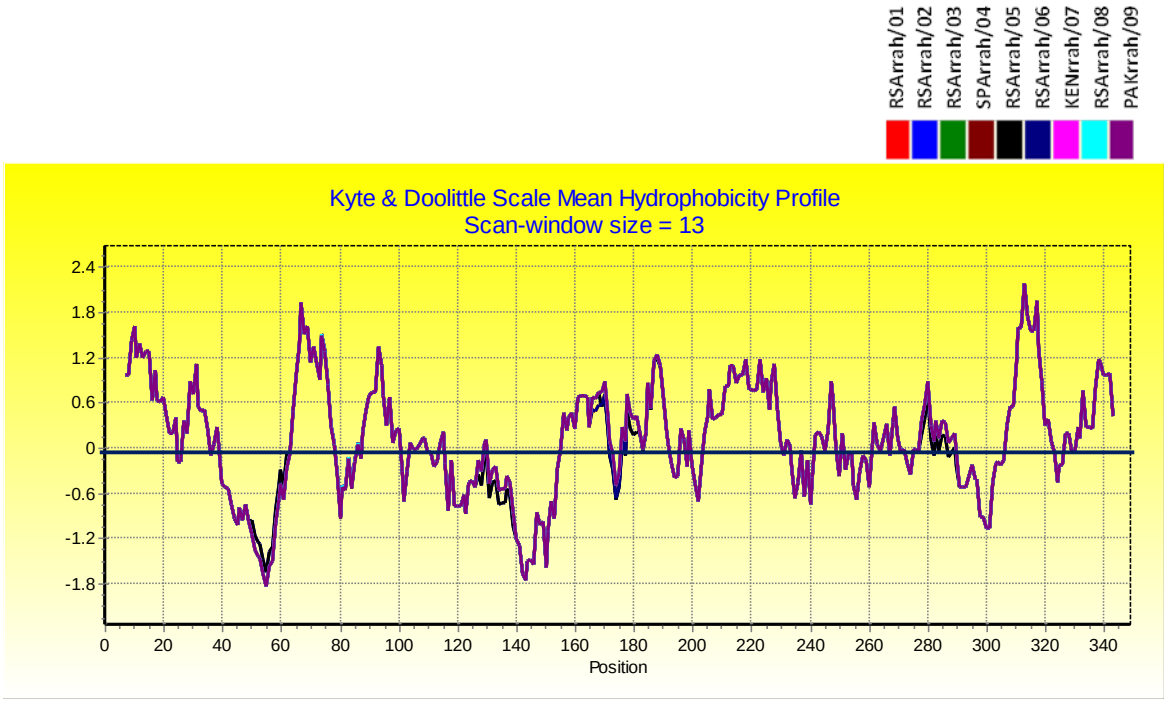


Figure 3.36: Hydrophobicity plot of the AHSV VP7 protein.
The hydrophobicity plot was inferred using Kyte and Doolittle Hydrophobicity Scale (Kyte and Doolittle, 1982) accessed through BioEdit (Hall, 1999). Both hydrophobic and hydrophilic regions were predicted along VP7 amino acid sequence. Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0. Strains of different AHSV serotypes are represented by different coloured lines, as per key.

The high percentage aa identity between AHSV reference strains is reflected in the plot illustrating identities along the genome segment (Figure 3.35) where only scattered variations were identified. The ratio of non-synonymous (d_N) to synonymous (d_S) substitutions per site within the VP7 ORF indicates strong purifying selection (d_N/d_S = 0.017) (Table 3.22) with values comparable to other highly conserved proteins like VP1 and VP3. Comparable aa substitution rates were also detected in the coding sequence, at 5.6×10^{-2} nucleotide substitutions per site (Table 3.22).

Phylogenetic analyses of nt and aa sequences of Seg-8 / VP7 reflect the spit between closely related RSArrah/02 and RSArrah/05 and the distinct strongly interrelated group containing the remaining reference strains (Figure 3.37 and 3.38). Interestingly, the RSArrah/02 and RSArrah/05 strains that are closely related at the nucleotide level (99.9% identity) group more distantly at the aa level (98% identity). However, the opposite was observed for SPArrah/04, KENrrah/07 and RSArrah/08 (Table 3.17) which share >97.3% nt identity, but encode identical VP7 aa sequences (Table 3.17). This suggests different evolutionary pressures playing a role on Seg-8 in the two groups of isolates.

Phylogenetic trees illustrating relatedness within nucleotide and amino acid sequences of Seg-8 were generated using ML methods (Felsenstein, 1981). A NJ method (Saitou & Nei, 1987) was also used increasing confidence in the analysis (data not shown).

Table 3.16: Genetic information for Seg-8 of nine AHSV reference strains.

AHSV isolate name	Segment length (bp)	Open reading frame (bp)	G+C content (%)	Protein size (aa)	Predicted protein mol mass (kDA)	Stop codon	5' Terminal sequence	3' Terminal sequence
RSArrah/01	1167	18-1067	46.4	349	37.8	TAG	GTTTAA…	…ACCTAC
RSArrah/02	1167	18-1067	46.1	349	37.8	TAG	GTTTAT…	…ACTTAC
RSArrah/03	1167	18-1067	46.5	349	37.8	TAG	GTTAAA…	…ACCTAC
SPArrah/04	1167	18-1067	46	349	37.8	TAG	GTTTAA…	…ACCTAC
RSArrah/05	1167	18-1067	46.2	349	37.8	TAG	GTTTAA…	…ACCTAC
RSArrah/06	1167	18-1067	46.4	349	37.8	TAG	GTTTAA…	…ACTTAC
KENrrah/07	1167	18-1067	46	349	37.8	TAG	GTTTAA…	…ACCTAC
RSArrah/08	1167	18-1067	46.5	349	37.8	TAG	GTTTAA…	…ACCTAC
PAKrrah/09	1167	18-1067	46.5	349	37.8	TAG	GTTAAA…	…ACCTAC

Differences in nucleotide sequences are highlighted in dark blue.

Table 3.17: Percentage identities between Seg-8 nucleotide and VP7 amino acid sequences.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	**98**	99.4	99.7	98.3	**100**	99.7	99.7	99.4
RSArrah/02	**89.1**	ID	**98**	98.3	99.7	**98**	98.3	98.3	98
RSArrah/03	98.2	89.5	ID	99.7	98.3	99.4	99.7	99.7	**100**
SPArrah/04	99.5	89.5	98.4	ID	98.6	99.7	**100**	**100**	99.7
RSArrah/05	89.2	99.9	89.5	89.5	ID	98.3	98.6	98.6	98.3
RSArrah/06	**100**	**89.1**	98.2	99.5	89.2	ID	99.7	99.7	99.4
KENrrah/07	98.7	89.5	97.9	99.1	89.5	98.7	ID	**100**	99.7
RSArrah/08	97.6	**89.1**	98	97.8	89.2	97.6	97.3	ID	99.7
PAKrrah/09	98.2	89.5	**100**	98.4	89.5	98.2	97.9	98	ID

Identity levels in the nt (white area) and aa (blue shaded area) sequences of AHSV Seg-8 / VP7 were analysed for the nine reference strains. "ID" stands for identical sequences. Figures in bold highlight minimum percentage identity between strains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

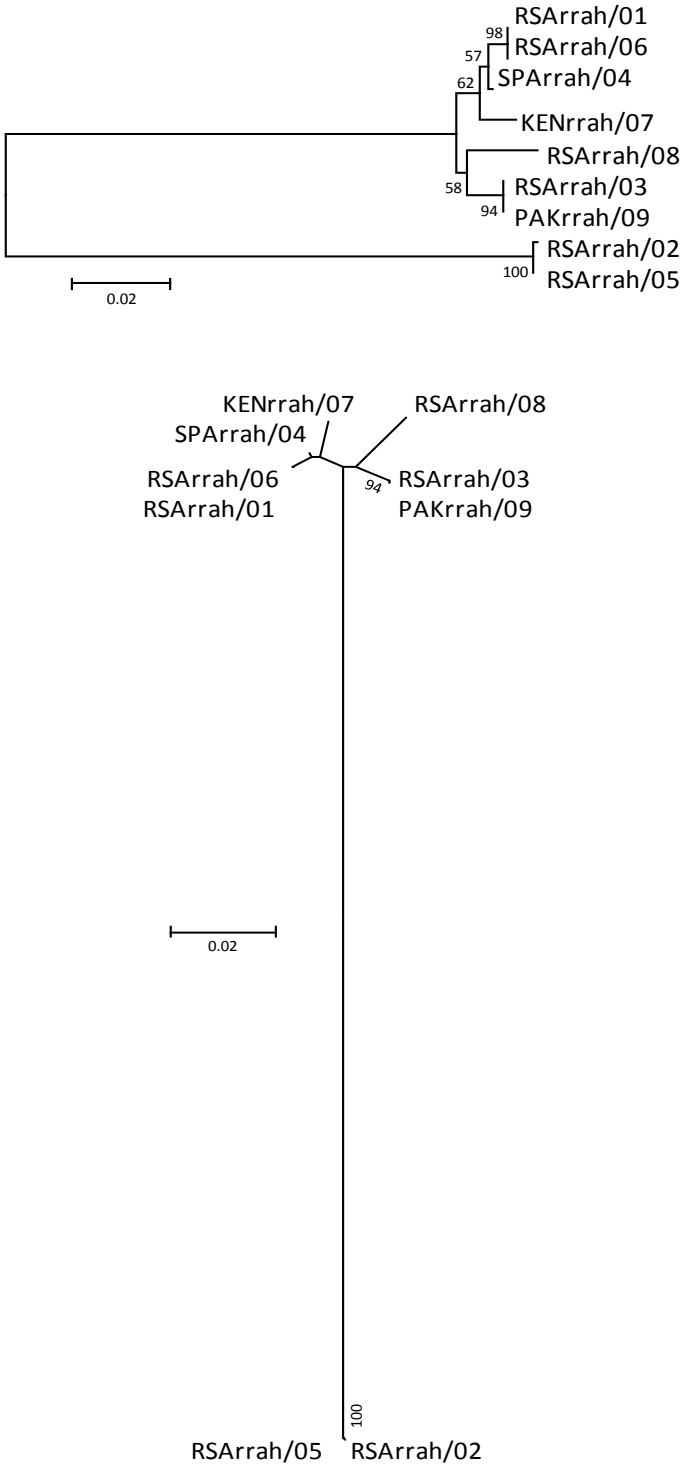


Figure 3.37: Phylogenetic analysis of Seg-8 nucleotide sequences.
Phylogenetic analyses of nt sequences of Seg-8 from the AHSV reference strains using ML method based on the Kimura 2-parameter model (K2) (Kimura, 1980) with a discrete Gamma distribution (rectangular (top) and radial trees(bottom)). The trees are drawn with branch lengths measured by the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

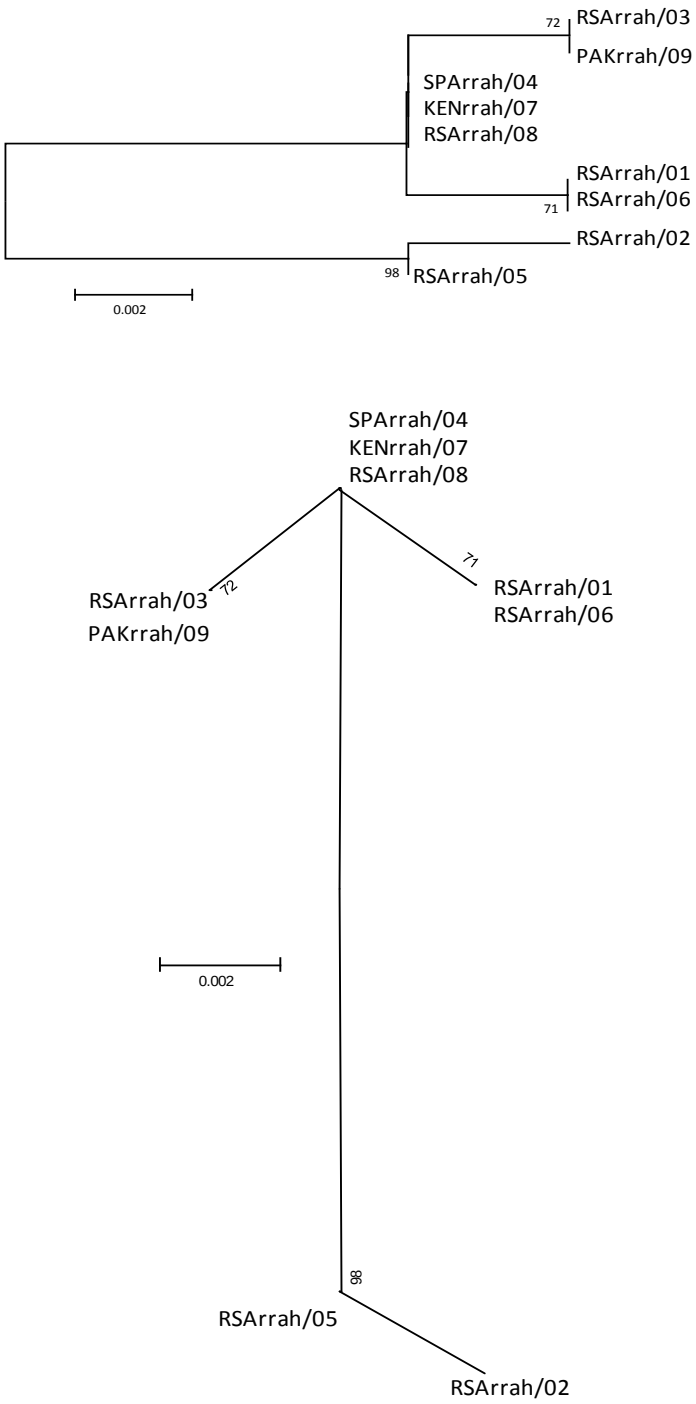


Figure 3.38: Phylogenetic analysis of VP7 amino acid sequences.
Phylogenetic analysis of aa sequences of VP7 from the nine AHSV reference strains are presented as rectangular (top) and radial (bottom) trees. The analysis uses a ML method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992) with a discrete Gamma distribution. The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

3.2.3.9: Genome segment 9

AHSV Seg-9 has a G+C content of 44.1 to 44.9 5 and is 1166 bp long in seven out of the nine AHSV reference strains, but 1165 bp in RSArrah/03 and PAKrrah/09 (Table 3.18). These variations in lengths are due to a single nucleotide deletion within the 5' NCR, which for most of strains investigated is 22 bp long, but of 21 bp in RSArrah/03 and PAKrrah/09 (Table 3.18). The 5' terminal hexanucleotide sequence of RSArrah/03 and PAKrrah/09 (5'-GTTAAA...3') differs from the remaining reference strains (5'-GTTTAA...3') (Table 3.18). The, 3' NCR of Seg-9 is 46 nt in length for all of the reference stains and is fully conserved between them, with the following 3' hexanucleotide sequence: 5'…...ACATAC-3' (Table 3.18). Identity levels of > 96.7% overall were detected in Seg-9 of the reference strains, showing that that Seg-9 is highly conserved between these strains (Table 3.19). RSArrah/01 and RSArrah/06 as well as RSArrah/05 and RSArrah/08 contain identical Seg-9 sequences, indicating common ancestry and the occurrence of reassortant events during the evolution of these strains (Table 3.19). Graphical representation of nt identities shows a low level of variation over the whole length of Seg-9, with more conserved regions at both ends (Figure 3.39).

AHSV Seg-9 codes for non-structural protein NS2 from a single, 1098nt ORF (Table 3.18), encoding the 365 aa NS2 protein, which has a molecular mass of 41 kDa (Table 3.18). The protein is mainly hydrophilic but with some hydrophobic regions (Figure 3.40). Pair-wise identities for the strains investigated were >98.1% (Table 3.19) with three strain pairs (RSArrah/01 and RSArrah/06; RSArrah/05 and RSArrah/08; RSArrah/02 and SPArrah/04) expressing identical versions of NS2 (Table 3.19). The first two pairs also contain fully identical NS9 sequences (Table 3.19), again indicating a

common ancestry and the occurrence of reassortment events during the evolution of these strains (Table 3.19).

Graphical representation of the aa sequence identities among the AHSV nine reference strains shows infrequent regions with low level variation along the protein (Figure 3.39). The ratio of non-synonymous (d_N) to synonymous (d_S) substitutions per site of d_N/d_S = 0.052 indicating that purifying selection is taking place on the NS2 protein coding sequence with a substitution rate of 2.5 x 10^{-2} (Table 3.22).

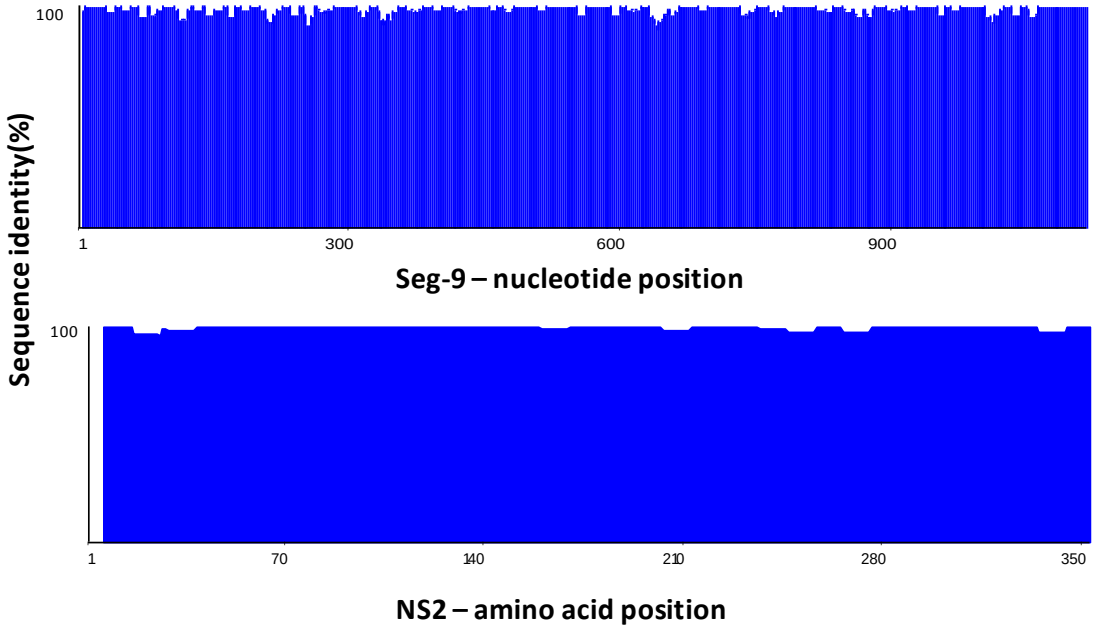


Figure 3.39: Graphical representation of nucleotide and amino acid sequences identities of AHSV Seg-9/NS2.
A low level of variability was detected in the nt sequence of Seg-9, which translates to an even smaller number of changes at the aa level in NS2. Alignments and plots were constructed using VectorNTI software (Invitrogen); analytical window size 10.

Phylogenetic analyses of Seg-9 and NS2 shows a close relationship between the nine reference strains (Figure 3.41 and 3.42). Nucleotide distances between RSArrah/02 and

SPArrah/04 (Table 3.19 and Figure 3.41) was eliminated in the aa sequences of NS2, as reflected in the topology of aa phylogenetic tree (Figure 3.42). Interestingly, despite both RSArrah/03 and PAKrrah/09 sharing identical 5' NCR, which are different to the remaining strains, these two viruses group separately (due to differences in the ORF) in both nt and aa based phylogenies (Figures 3.35 and 3.36).

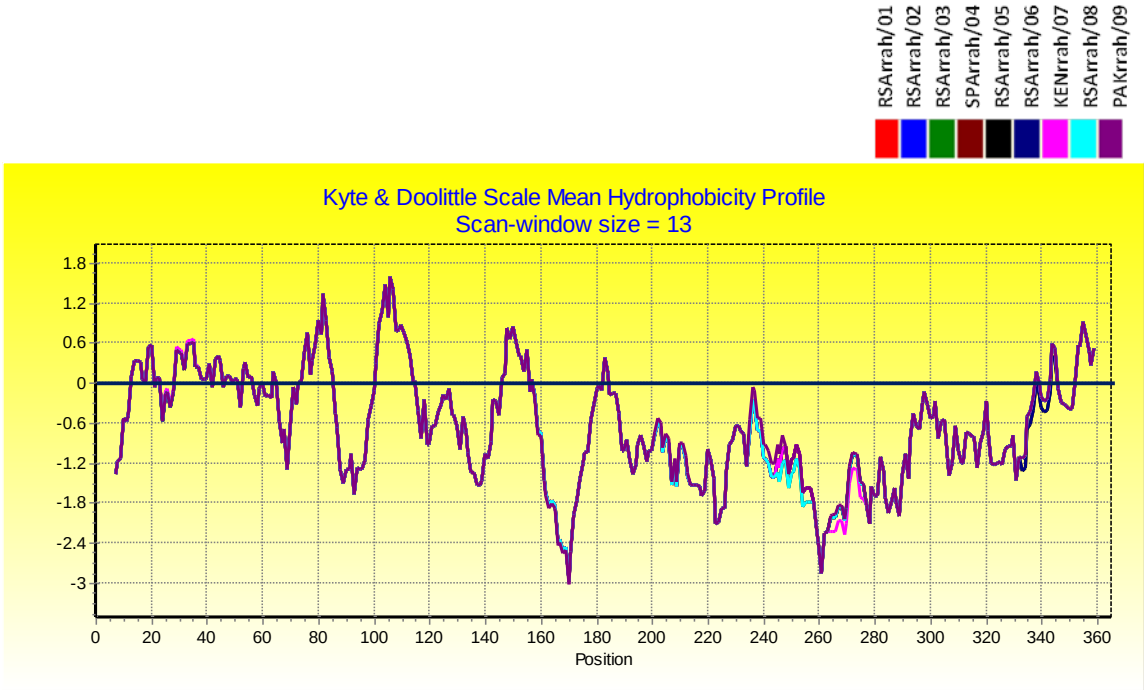


Figure 3.40: Hydrophobicity plot of the AHSV NS2 protein.
The hydrophobicity plot for AHSV NS2 was inferred using a Kyte and Doolittle Hydrophobicity Scale (Kyte and Doolittle, 1982) accessed through BioEdit (Hall, 1999). NS2 was shown to be mainly hydrophobic with some hydrophilic regions. Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0. Strains of different AHSV serotypes are represented by different coloured lines, as per key.

The nt and aa acid trees presented for Seg-9 / NS2 were constructed using a ML (Felsenstein, 1981) and NJ methods (data not shown) (Saitou & Nei, 1987), which produced comparable results.

Table 3.18: Genetic information for Seg-9 / NS2 of nine reference strains.

AHSV isolate name	Segment length (bp)	Open reading frame (bp)	G+C content (%)	Protein size (aa)	Predicted protein mol mass (kDA)	Stop codon	5' Terminal sequence	3' Terminal sequence
RSArrah/01	1166	23-1120	44.5	365	41	TGA	GTTTAA…	…ACATAC
RSArrah/02	1166	23-1120	44.9	365	41	TGA	GTTTAA…	…ACATAC
RSArrah/03	**1165**	**22-1119**	44.5	365	41	TGA	GTTAAA…	…ACATAC
SPArrah/04	1166	23-1120	44.1	365	41	TGA	GTTTAA…	…ACATAC
RSArrah/05	1166	23-1120	44.2	365	41	TGA	GTTTAA…	…ACATAC
RSArrah/06	1166	23-1120	44.5	365	41	TGA	GTTTAA…	…ACATAC
KENrrah/07	1166	23-1120	44.5	365	41	TGA	GTTTAA…	…ACATAC
RSArrah/08	1166	23-1120	44.2	365	41	TGA	GTTTAA…	…ACATAC
PAKrrah/09	**1165**	**22-1119**	44.9	365	41	TGA	GTTAAA…	…ACATAC

Differences in the length between strains are indicated by bold face in blue. Nucleotide differences are highlighted in dark blue.

Table 3.19: Percentage identities between Seg-9 nucleotide and NS2 amino acid sequences.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	99.5	**100**	99.5	99.2	**100**	98.9	99.2	**98.1**
RSArrah/02	98.5	ID	99.5	**100**	99.7	99.5	99.5	99.7	98.6
RSArrah/03	99.9	98.4	ID	99.5	99.2	**100**	98.9	99.2	**98.1**
SPArrah/04	97.8	97.9	97.7	ID	99.7	99.5	99.5	99.7	98.6
RSArrah/05	97.7	97.9	97.6	99.1	ID	99.2	99.2	**100**	98.4
RSArrah/06	**100**	98.5	99.9	97.8	97.7	ID	98.9	99.2	**98.1**
KENrrah/07	97.1	97.3	97	97.1	97.2	97.1	ID	99.2	98.4
RSArrah/08	97.7	97.9	97.6	99.1	**100**	97.7	97.2	ID	98.4
PAKrrah/09	**96.7**	97	**96.7**	97	97.1	**96.7**	96.8	97.1	ID

Identity levels in the nt (white area) and aa (blue shaded area) sequences of AHSV Seg-9/ NS2 were analysed for the nine reference strains. "ID" stands for identical sequences. Figures in bold highlight minimum percentage identity between strains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

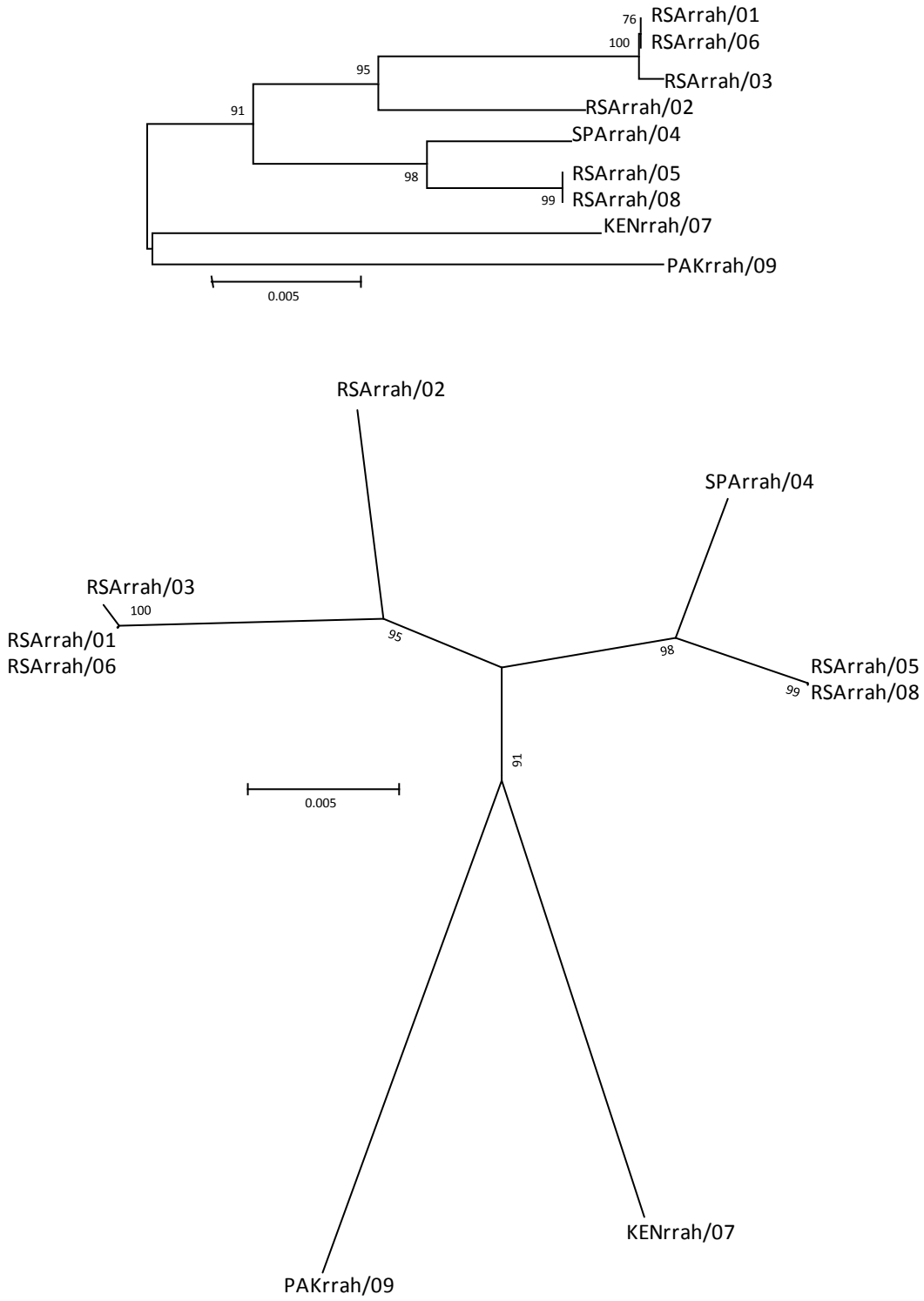


Figure 3.41: Phylogenetic analysis of AHSV Seg-9 nucleotide sequences.
Phylogenetic analyses of nt sequences of Seg-9 from the AHSV reference strains using ML method based on based on the Tamura-Nei model (TN93) (Tamura & Nei, 1993) (rectangular (top) and radial trees(bottom)). The trees are drawn with branch lengths measured by the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

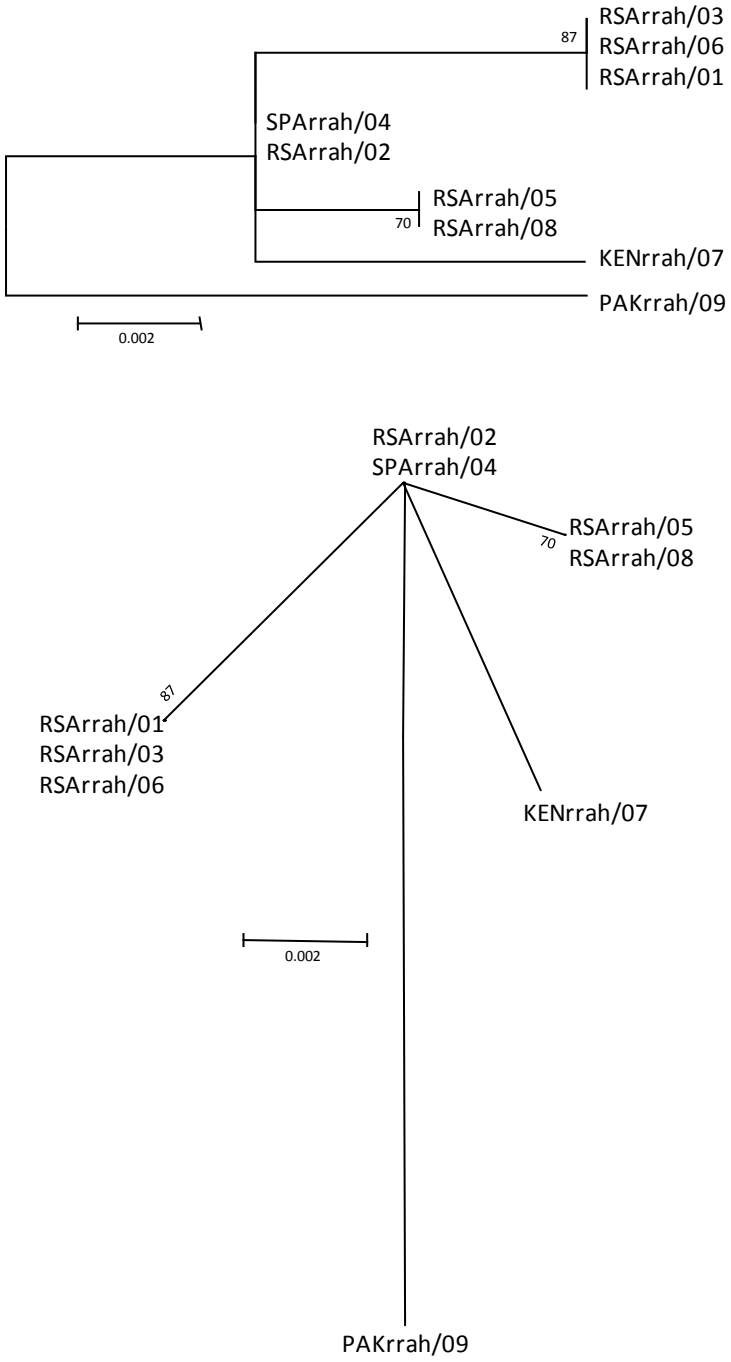


Figure 3.42: Phylogenetic analysis of AHSV NS2 amino acid sequences.
The phylogenetic analysis of NS2 amino acid sequences of nine reference strains of AHSV are presented as rectangular (top) and radial (bottom) trees. The analysis was inferred by using ML method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992). The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

3.2.3.10: Genome segment 10

The length of Seg-10 from the nine reference strains of AHSV varies between 756 to 763 bp, with a G+C content that ranges from 42 to 45, the widest variation in any of the genome segments analysed (Table 3.20). The 5' NCR is 19-20 nt and is > 94.4% conserved, with 5'-GTT$^{A}/_{T}$AA...3' terminal hexanucleotide sequences (Table 3.2). The 3' NCR is more variable with only >71.8% conservation and varies in length between 74 nt for most of the reference strains, but 88 nt for RSArrah/01 and RSArrah/02 and 85 nt for RSArrah/03 and KENrrah/07. Despite these differences the 3' hexanucleotide of Seg-10 is conserved for all of the reference strains as 5'...ACTTAC-3' (Table 3.20).

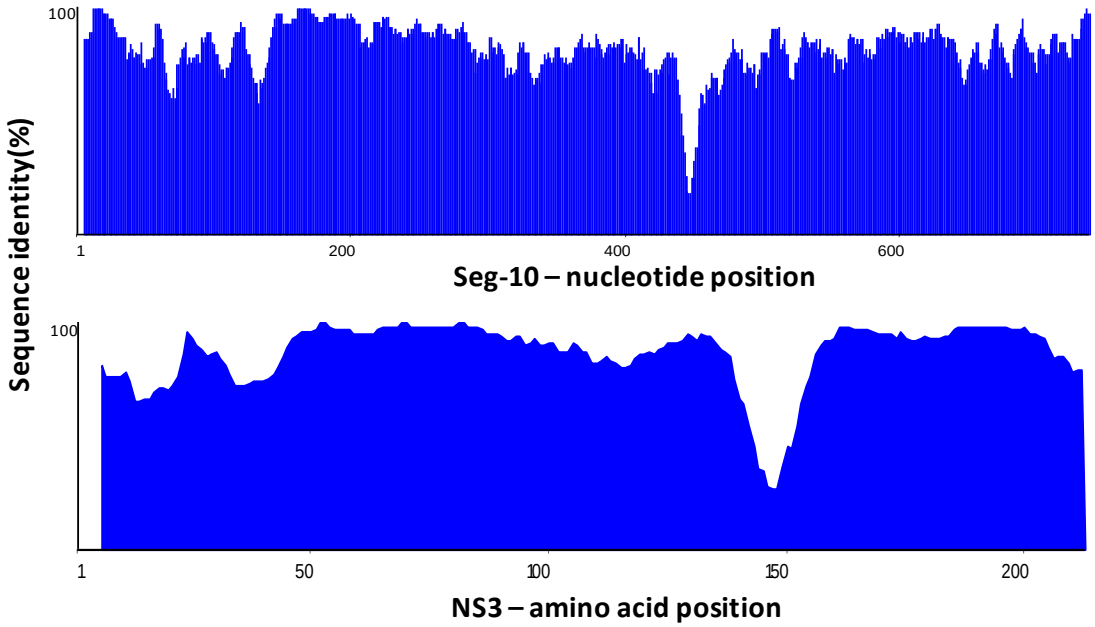


Figure 3.43: Graphical representation of nucleotide and amino acid identity levels for AHSV Seg-10/NS3.
Seg-10 and NS3 sequence are variable throughout the alignments between the strains investigated, with increased variation between sequences at the 5'/N-terminal ends and in the central part, particularly around aa 150. Alignments and plots were constructed using VectorNTI software (Invitrogen); analytical window size 10.

Overall Seg-10 shows >67.9% nt identity and within this limited group of viruses is therefore the second most variable region of the AHSV genome, after Seg-2, (Table 3.21) (see Section 3.2.3.2). The level of nt identity is not uniform across the whole of Seg-10, with clear regions of higher and lower variability (Figure 3.43).

The large ORF of AHSV Seg-10, which is 657 bp in RSArrah/01 and RSArrah/02, but 654 bp in the remaining isolates, uses the same stop codon 'TAG' in each isolate (Table 3.20). AHSV Seg-10 codes for 'NS3', a membrane protein which is 219 or 218 aa long (depending on the length of Seg-10) with a predicted molecular mass of 23.6 to 23.8 kDa (Table 3.20) (O'Hara *et al.*, 1998; van Staden *et al.*, 1995). NS3a is encoded from the same ORF as NS3, but using a 2nd start codon, which translates into a protein of 207-209 amino acids with a predicted molecular mass of 22.3-22.6 kDa (Table 3.20).

The NS3/NS3a proteins vary significantly between most of the AHSV isolates investigated in this study, with identity levels of > 66% (for NS3). The strains RSArrah/01 and RSArrah/02 encode identical versions of NS3/N3a (Table 3.21). Out of the eleven distinct proteins encoded by AHSV, only outer-capsid protein VP2 of the nine reference strains (Section 3.2.3.2) shows a higher level of variability than NS3. A plot of percentage identities in Seg-10/NS3/NS3a, for all of the reference strains investigated, shows higher variability in the N-terminal region of the protein (which includes the first 10 aa – the region of NS3 that is missing in NS3a) and again between aa 130-160 (Figure 3.43). The 2nd variable region has previously been mapped as exterior of the cell membrane (van Staden *et al.*, 1995). Two hydrophobic regions, flanking the stretch of variable amino acids, were identified in NS3 from all of the strains investigated (Figure 3.44), which represent trans-membrane helices (Bansal *et al.*, 1998; van Staden *et al.*,

1995). The levels of aa identity were calculated as > 66% for NS3, which is slightly less than the level nucleotide identity in Seg-10 (>67.9%) (Table 3.21). However, inferred non-synonymous (d_N) to synonymous (d_S) substitutions per site (d_N/d_S = 0.222) indicates an overall purifying selection on the Seg-10 gene with values similar to those calculated for Seg-2/VP2 and Seg-4/NS4 (Table 3.22). The nucleotide substitution rate within Seg-10 gene of 1.16 substitutions per site is comparable to Seg-2, encoding VP2 (Table 3.22). This observation points to these two as the fastest mutating regions of the AHSV genome.

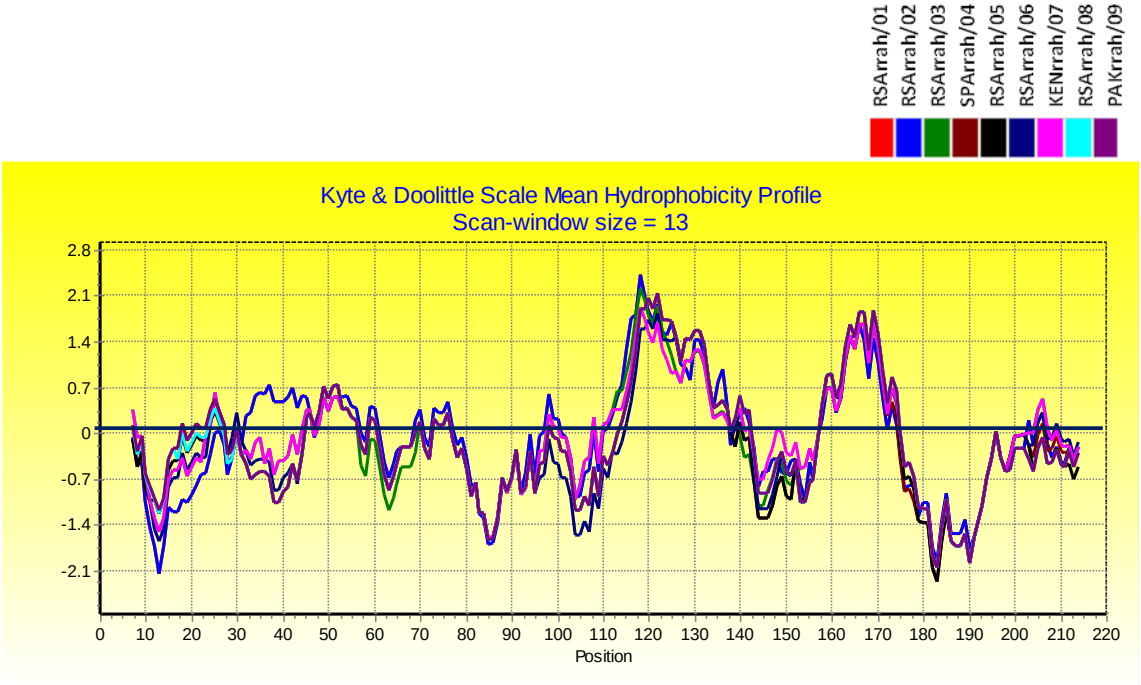


Figure 3.44: Hydrophobicity plot of the NS3 protein.
The hydrophobicity plot for AHSV NS3 was inferred using a Kyte and Doolittle Hydrophobicity Scale (Kyte and Doolittle, 1982) accessed through BioEdit (Hall, 1999). NS3 was shown to be mainly hydrophilic with two clear hydrophobic regions. Hydrophobic regions of the protein are indicated as peaks above 0, while hydrophilic regions are indicated as troughs below 0. Strains of different AHSV serotypes are represented by different coloured lines, as per key. Some differences in the hydrophobicity profiles of individual isolates are visible on the graph, especially for RSArrah/02 (dark blue plot) in the n-terminal part of the protein. The significance of this variation is uncertain.

Phylogenetic analyses of the nt and aa sequences for Seg-10 / NS3 showed the same clustering of the nine reference strains into, three distinct phylogenetic lineages: α, β and γ as reported previously (Figure 3.45 and 3.46) (Martin et al., 1998; Quan et al., 2008; Sailleau et al., 1997; van Niekerk et al., 2003). Cluster α, includes five reference strains (SPArrah/04, RSArrah/05, RSArrah/06 RSArrah/08 and PAKrrah/09) with aa identities > 91.1% between isolates (Figure 3.46, Table 3.21). Reference strains RSArrah/03 and KENrrah/07 show 97.7% identity and group in NS3 cluster β. Cluster γ includes RSArrah/01 and RSArrah/02, which encode NS3 proteins with identical aa sequences (98.3% nucleotide identity) of (Figure 3.46, Table 3.21).

Strains grouping in clusters β (RSArrah/03 and KENrrah/07) and γ (RSArrah/01 and RSArrah/02) are also more closely related to each other than to the remaining isolates in Seg-2 (Section 3.2.3.2, Figure 3.11). The grouping of strains in cluster α, does not correlate with their serotype, and Seg-2 grouping (Section 3.2.3.2, Figure 3.11).

Phylogenetic trees are presented showing ML analyses (Felsenstein, 1981) or Seg-10/NS3 of the nine reference strains (Figure 3.45 and 3.46). Trees were also created using NJ method (Saitou & Nei, 1987) showing similar topology (data not shown).

Table 3.20: Genetic information for Seg-10 of nine AHSV reference strains.

AHSV isolate name	Segment length (bp)	NS3 ORF (bp)	NS3 G+C content (%)	NS3 protein size (aa)	NS3 predicted protein mol mass (kDA)	NS3a ORF (bp)	NS3a protein size (aa)	NS3a predicted protein mol mass (kDA)	Stop codon	5' Terminal sequence	3' Terminal sequence
RSArrah/01	763	19-675	44.6	219	23.8	49-675	209	22.6	TAG	GTTTAA…	…ACTTAC
RSArrah/02	763	19-675	45	219	23.8	49-675	209	22.6	TAG	GTTTAA…	…ACTTAC
RSArrah/03	758	20-673	44.9	218	23.7	50-673	208	22.4	TAG	GTTTAA…	…ACTTAC
SPArrah/04	756	19-672	43.5	218	23.7	52-672	207	22.5	TAG	GTTTAA…	…ACTTAC
RSArrah/05	757	20-673	43.9	218	23.6	53-673	207	22.5	TAG	GTTAAA…	…ACTTAC
RSArrah/06	757	20-673	43.6	218	23.7	53-673	207	22.6	TAG	GTTAAA…	…ACTTAC
KENrrah/07	757	19-672	45	218	23.6	52-672	207	22.3	TAG	GTTTAA…	…ACTTAC
RSArrah/08	756	19-672	43	218	23.6	52-672	207	22.5	TAG	GTTTAA…	…ACTTAC
PAKrrah/09	756	19-672	42	218	23.6	52-672	207	22.5	TAG	GTTAAA…	…ACTTAC

Nucleotide differences between strains are highlighted in dark blue.

Table 3.21: Percentage identities between Seg-10 nucleotide and NS3 amino acid sequences.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArrah/01	ID	**100**	**66**	67.3	67.8	67.9	**66**	68.2	68.2
RSArrah/02	98.3	ID	**66**	67.3	67.8	67.9	**66**	68.2	68.2
RSArrah/03	68.2	68	ID	75.7	75.7	74.9	97.7	75.2	76.2
SPArrah/04	68.4	68.3	75.7	ID	98.6	90.2	75.7	96.3	96.3
RSArrah/05	68.7	68.5	75.4	**99.2**	ID	90.2	75.7	95.8	95.8
RSArrah/06	68.1	68.1	73.8	87.2	87.2	ID	75.3	90.7	91.1
KENrrah/07	68	**67.9**	98.5	75.5	75.3	73.9	ID	75.2	76.2
RSArrah/08	68.3	68	75.9	96.6	96.3	87.6	76.1	ID	98.6
PAKrrah/09	68	68	75.8	95.8	95.8	88.6	75.9	97.6	ID

Identity levels in the nt (white area) and aa (blue shaded area) sequences of AHSV Seg-10/ NS3 were analysed for the nine reference strains. "ID" stands for identical sequences. Figures in bold highlight minimum percentage identity between strains analysed and bold underlined figures emphasise values of maximum percentage identity. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

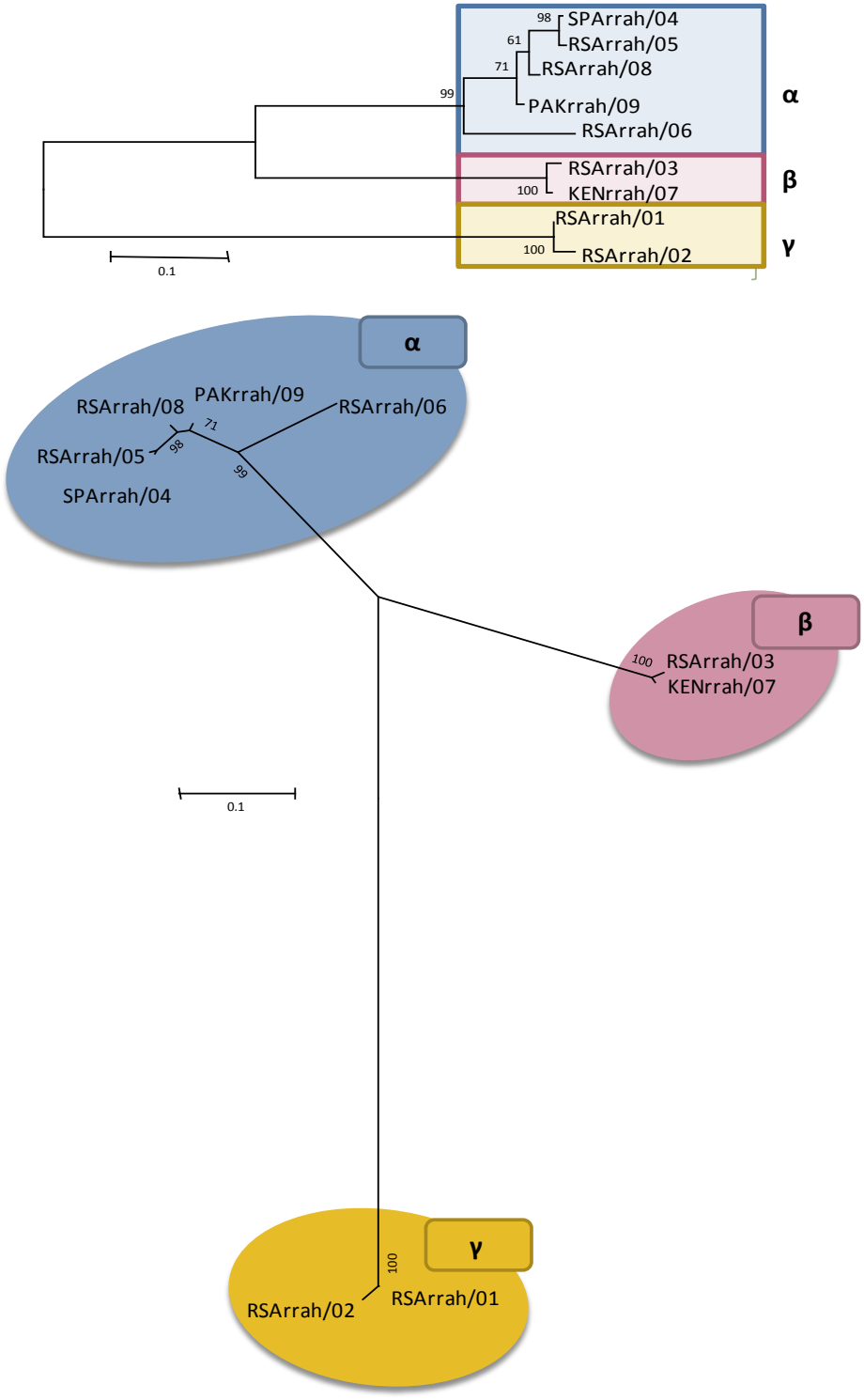


Figure 3.45: Phylogenetic analysis of Seg-10 nucleotide sequences.
Phylogenetic analyses of nt sequences of Seg-10 from the AHSV reference strains using ML method based on the Hasegawa-Kishino-Yano model (HKY) (Hasegawa *et al.*, 1985) (rectangular (top) and radial trees(bottom)) with a discrete Gamma distribution. The trees are drawn with branch lengths measured by the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

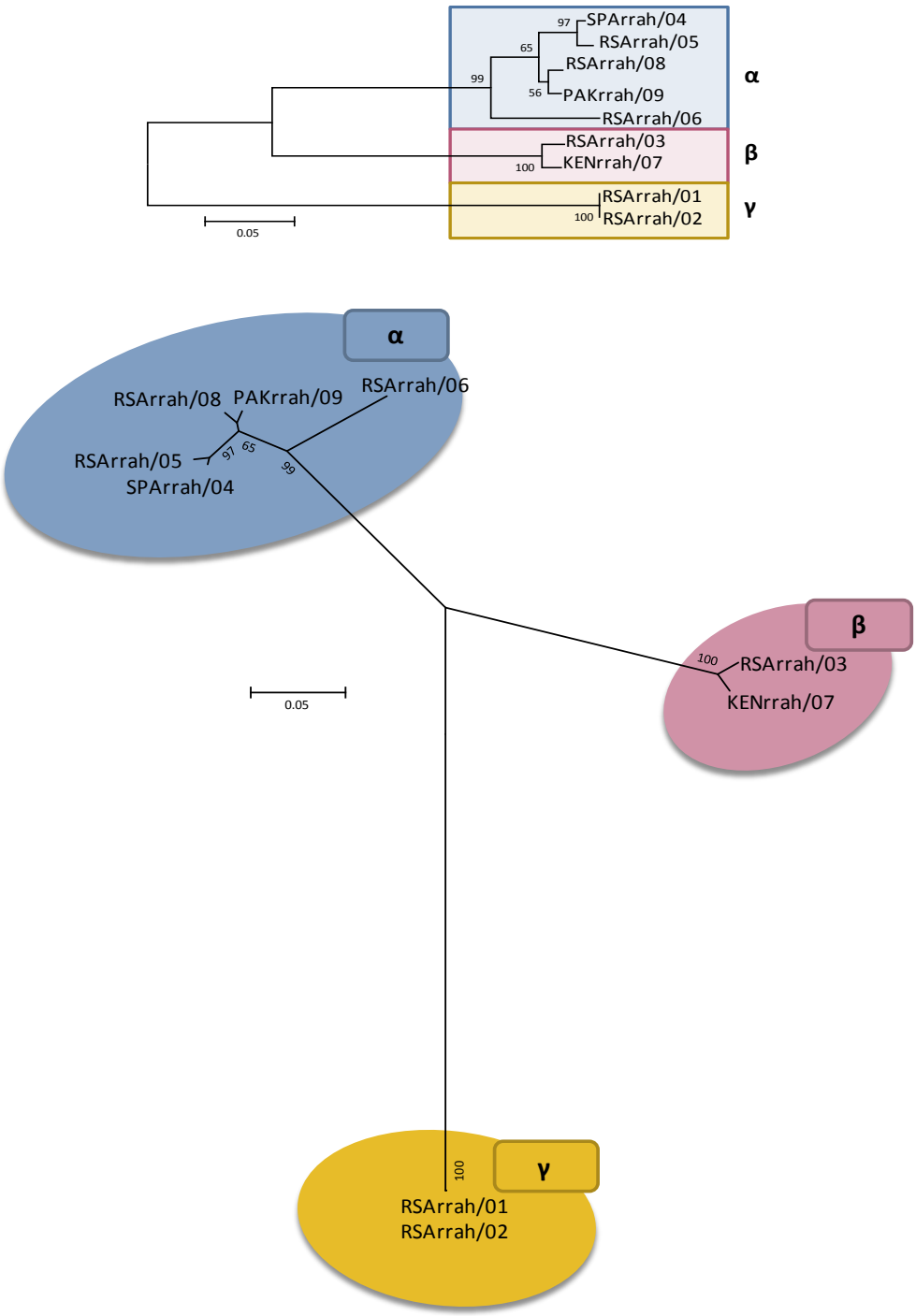


Figure 3.46: Phylogenetic analysis of NS3 amino acid sequences.
The phylogenetic analysis of NS3 amino acid sequences of nine reference strains of AHSV are presented as rectangular (top) and radial (bottom) trees. The analysis used ML method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992) with a discrete Gamma distribution. The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

3.3: Discussion

The work described in this Chapter provides the first whole genome, full length sequence database for the nine historical reference strains of AHSV representing each of the known serotypes of AHSV. During this comprehensive study nucleotide and amino acid sequences were analysed for each of the genome segment separately. Analyses provided information on levels of sequence identities and phylogenetic comparisons of nucleotide and amino acid sequences, identified variable regions within individual genome segments, estimated selection pressures on protein coding sequences and hydrophobicity profiles of encoded proteins.

This database forms bases for future molecular epidemiological studies of AHSV, including molecular characterisation of novel isolates and genome segment reassortments, similar to those described for BTV, EHDV and EEV (Aharonson-Raz *et al.*, 2011; Allison *et al.*, 2012; Maan *et al.*, 2008; Maan *et al.*, 2010b; Sailleau *et al.*, 2012). These data also provided basis for development of novel real-time RT-PCR assays for detection of AHSV and identification of its type, as described in Chapter 4.

As a result of sequence comparisons, different levels of nucleotide and amino acid identities were described for individual genome segments (Figure 3.47). High levels of sequence identity are evident in Seg-1, 3, 4, 7 and 8 (encoding AHSV core proteins VP1, VP3, VP4, VP6 and VP7), Seg-5, 9 and 7 (encoding AHSV non-structural proteins NS1, NS2 and NS4). The remaining AHSV genes, (including Seg-2 and Seg-6, encoding outer-capsid proteins VP2 and VP5 and the last of the AHSV non-structural protein gene 'Seg-10/NS3) are more variable, that is thought to reflect selective pressure by the hosts' immune system, particularly on VP2 and to a lesser extent on VP5. The extracellular

domain of NS3 in particular also shows a high degree of variability that could potentially also reflect interactions with the hosts' immune responses.

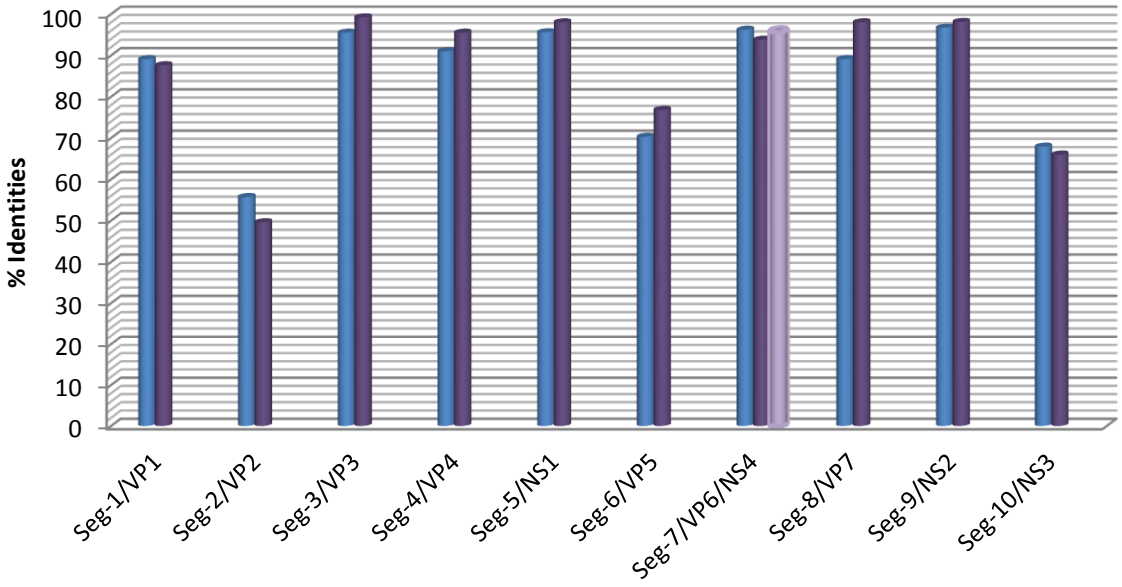


Figure 3.47: Minimum percentage identities levels for each AHSV genome segment. Blue bars represent t sequence identities. Purple bars represent aa sequence identities.

Table 3.22: Estimates of substitution rate and selection pressure for protein coding sequences of AHSV.

Genome segment number	substitution rate (subs/site)	d_N/d_S (95%) CI
Seg-1 (VP1)	0.0816	0.029
Seg-2 (VP2)	1.96	0.317
Seg-3 (VP3)	0.0368	0.012
Seg-4 (VP4)	0.06	0.067
Seg-5 (NS1)	0.033	0.067
Seg-6 (VP5)	0.34	0.064
Seg-7 (VP6)	0.021	0.883
Seg-7 (NS4)	0.028	0.289
Seg-8 (VP7)	0.056	0.017
Seg-9 (NS2)	0.025	0.052
Seg-10 (NS3)	1.16	0.222

Substitution rate values were estimated using single-likelihood ancestor-counting (SLAC) method (Kosakovsky Pond & Frost, 2005).

Nucleotide substitution rates were estimated for all of the AHSV protein coding sequences. However, as the investigated strains are historical, highly passaged and restricted in numbers, the ratio was expressed as number of nucleotides substitutions per site, rather than more commonly used "nucleotides substitutions per site per year" (Table 3.22).

3.3.1: The outer capsid proteins: VP2 and VP5

VP2: Analysis of Seg-2 confirmed that it is the most variable region between different AHSV types with 55.7-68.2% nt sequence identity and 49.3-77.2% aa identity, in VP2 between serotypes. This low sequence identity reflects the outer-most position of the protein on the virus particle, its function as a cell-attachment protein and interactions with neutralising antibodies (Manole *et al.*, 2012; Roy *et al.*, 1994; Zhang *et al.*, 2010). Consequently, VP2 is the primary determinant of virus type (Bentley *et al.*, 2000; Burrage & Laegreid, 1994; Castillo-Olivares *et al.*, 2011; Martinez-Torrecuadrada & Casal, 1995; Martinez-Torrecuadrada *et al.*, 2001; MartinezTorrecuadrada *et al.*, 1996; Vreede & Huismans, 1994).

As VP2 is exposed to immunological pressure from the hosts, changes in aa sequence that alter its serological identity may help the virus to escape neutralisation, providing a selective advantage for the virus. Lower minimum-levels of nt and aa sequence identities have been detected in VP2 from the members of other *Orbivirus* species, e.g. BTV (>49% and >27%, respectively) and EHDV (>45% and >31%, respectively) (Anthony *et al.*, 2009c; Maan *et al.*, 2007a). This could be related to the greater geographical distribution of the BTV and EHDV on different continents, in comparison to AHSV and therefore to

independent evolution of geographicaly separated lineages of the virus (topotypes), and greater numbers of isolates.

However, in each of these three *Orbivirus* species the lowest nt identity detected in VP2 is significantly greater than the lowest amino acid identity. This implies that in AHSV, BTV and EHDV many nt differences between strains are translated as non-synonymous aa changes. Despite this, purifying selection has been inferred to play part in the AHSV Seg-2 coding sequence (d_N/d_S=0.317) (Table 3.22) and BTV (d_N/d_S=0.122) (Carpi *et al.*, 2010), (d_N/d_S= 0.132) (Maan *et al.*, 2011b), suggesting significant structural and functional constraints on the protein.

A multiple alignment of VP2 aa sequence of the nine AHSV reference strains, showed that despite overall sequence variability (Figure 3.7) some features, including the VP2 hydrophobicity profile (Figure 3.9) appear to be conserved for all nine AHSV types. This also suggests that, despite differences in neutralising epitopes, regions important for maintaining the structure and function of VP2 are conserved.

VP2 aa sequence alignments also showed regions of low sequence identity between the strains investigated (Figure 3.7). Some of these are consistent with the antigenic regions previously identified in AHSV-3 (Bentley et al., 2000), AHSV-9 (Venter et al., 2000) and AHSV-4 (Martinez-Torrecuadrada et al., 2001) (Figure 3.48). PEPSCAN analysis, in combination with serological tests, identified two major antigenic sites in AHSV-4, between residues 223-400 and 568-681, as well as two neutralising epitopes between residues 321-339 and 377-400 (Martinez-Torrecuadrada et al., 2001). These epitopes are not only located in variable regions between all nine serotypes of AHSV but also in domains predicted as hydrophilic indicating surface location of these sites (Figures 3.7 and 3.9).

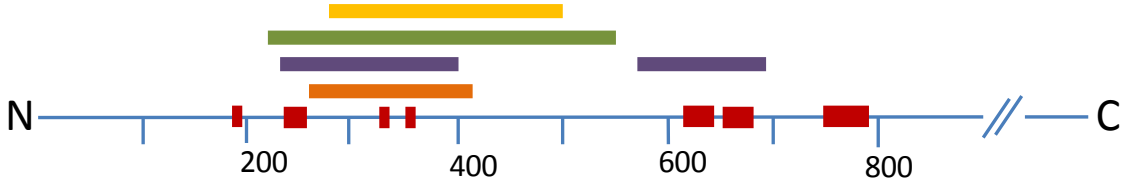


Figure 3.48: Graphical illustration of detected insertion / deletion sites in VP2 in relation to already identified antigenic sites.
The blue line represents linear VP2 protein with identified insertions / deletions sites between analysed strains (as presented in details in Figure 3.8) indicated as red blocks. Several antigenic regions have been identified previously to be scattered along the N-terminal part of the proteins as indicated by different coloured lines: orange (254-414 aa in AHSV-4) (Martinez-Torrecuadrada & Casal, 1995), purple (223-400 and 568-681 in AHSV-4) (Martinez-Torrecuadrada *et al.*, 2001), green (224-543 in AHSV-3) (Bentley *et al.*, 2000) and in yellow (282-498 in AHSV-9) (Venter *et al.*, 2000b).

Interestingly, to achieve best-fit alignment in regions of low aa sequence identity between the nine AHSV types, several gaps were required in the sequences (Figure 3.8). Similar, serotype specific 'gaps' in multiple aa sequence alignment were identified in VP2 of BTV (Maan *et al.*, 2007a). Although these insertion / deletion regions in AHSV VP2 are serotype-specific, they also show considerable similarity between the pairs of AHSV serotypes that were reported to cross-neutralise (AHSV serotypes 1 and 2; 3 and 7; 5 and 8; 6 and 9) (Coetzer & Guthrie, 2004). Most of these insertion / deletion regions were found to be located in hydrophilic regions of VP2 (Figure 3.9) suggesting that they may be exposed on the surface of the outer capsid and some of them overlapped with antigenic sites / neutralising epitopes already identified (e.g. aa 568 to 681, and 321 to 339) (Martinez-Torrecuadrada et al., 2001) (Figure 3.48). This indicates that the antigenic sites described for AHSV-3, 4 and 9 are likely to be type specific. It also suggests that the insertion / deletion regions between aa 190 to 195 and 765 to 785 (Figure 3.8), which had not previously been identified as significant antigenic sites, may also be part of continuous or discontinuous neutralisation epitopes (Figure 3.48). The previously

reported serological-cross-relatedness of some pairs of AHSV serotypes may be related to similarities in these insertion/deletion sites and consequently their neutralising epitopes.

Phylogenetic comparison of the nine reference strains of AHSV shows agreement between sequence variation of Seg-2/VP2 sequences and AHSV serotype (Figure 3.10 and 3.11) confirming the importance of VP2 as its major determinant. These finding agree with previous studies concerning the level of Seg-2 sequence identity between different AHSV serotypes (Potgieter *et al.*, 2003; Williams *et al.*, 1998). These conclusions are also supported by the results of cross-hybridization study, which showed that Seg-2 of AHSV-3 hybridizes in a serotype specific manners (Bremer *et al.*, 1990).

It has been reported that some cross-neutralisation can occur between AHSV serotypes 1 and 2; 3 and 7; 5 and 8; 6 and 9 (Coetzer & Guthrie, 2004). Nucleotide and amino acid sequences of these serotype pairs were found in each case to be more closely related to each other than to the other serotypes (Figure 3.10 and 3.11; Table 3.4) confirming this serological observation.

VP5: The smaller of the AHSV outer-capsid proteins 'VP5' is encoded by Seg-6, which shows >70.3% nt and >76.8% aa identity between the nine AHSV reference strains and therefore represents another 'variable' segment of the virus genome (Figure 3.47, Table 3.22). VP5 is more inwardly located and to some extent may be shielded from the host's immunological pressures by VP2 (Nason *et al.*, 2004; Zhang *et al.*, 2010). This is consistent with higher levels of nt and aa sequence identity between AHSV serotypes, in Seg-6/VP5 than in Seg-2/VP2.

The level of nt and aa identity in Seg-6/VP5 of BTV (>56.7% and >54.2 % respectively,) and EHDV (>61.3% and >60.5% respectively) are lower than in AHSV (Anthony *et al.*,

2009c; Maan *et al.*, 2010b) which, as discussed for Seg-2/VP2, may reflect the analyses of more strains from a wider geographic distribution (that includes different topotypes).

Unlike Seg-2, the maximum variability within the Seg-6 nt sequence of the AHSV reference strains is greater than that of aa sequence (Table 3.12 and Figure 3.47) indicating that many of the nt substitutions are synonymous. Therefore, conservation of the VP5 function and/or structure is necessary for the viability of the virus. This observation is supported by estimated strong purifying (negative) selection of the VP5 coding sequence (d_N/d_S =0.064) despite a relatively high nucleotide substitution rate in comparison to other AHSV genome segments (3.4 x 10^{-1} substitutions per site) (Table 3.22). The d_N/d_S ratio calculated for BTV is of similar value (d_N/d_S=0.051) (Maan *et al.*, 2011b) suggesting similar selective pressures on this protein.

The phylogenetic analysis of AHSV Seg-6 / VP5 sequences (Figure 3.27 and 3.28) shows some resemblance the grouping of Seg-2/VP2 (Figure 3.10 and 3.11). However, unlike Seg-2, ML and NJ comparisons grouped Seg-6/VP5 of RSArrah/03, RSArra/06 and RSArrah/09 into a common, closely related cluster, with RSArrah/07 in a separate cluster (Figure 3.27 and 3.28). Also RSArrah/05 and RSArrah/08, despite belonging to different serotypes, shared the same version of Seg-6, (Figure 3.27 and 3.28; Table 3.12). Phylogenetic investigations of BTV and EHDV nt and aa sequences also show that some isolates with divergent VP2 (different serotypes) use similar version of VP5 (Anthony *et al.*, 2009c; Maan *et al.*, 2010b).

VP2 is regarded as the prime determinant of a serotypes in AHSV, BTV and EHDV (Bremer *et al.*, 1990; Huismans & Erasmus, 1981) and can elicit neutralizing antibodies protecting horses from homologous AHSV challenge (Roy *et al.*, 1996; Scanlen *et al.*, 2002; StoneMarschat *et al.*, 1996). In some cases neutralizing epitopes were also shown

to be located on VP5 of AHSV and Kemerovo viruses (Martinez-Torrecuadrada *et al.*, 1999; Moss *et al.*, 1987) but, unlike VP2, VP5 of BTV was not capable of inducing neutralising antibodies on its own (Marshall & Roy, 1990).

However, similarities in the phylogenetic topologies of Seg-2 and Seg-6 are evident for several different orbiviruses. It has also been shown that two reassortant viruses containing VP2 and VP5 originating from two distinct parental strains of BTV (BTV-3 and BTV-10 or BTV-20 and 21 respectively) raised antibodies that neutralised both of the parental strains (Cowley & Gorman, 1989; Mertens *et al.*, 1989b) indicating that both of the outer capsid proteins, when present in the same virus, can have an input on the conformation of the neutralising epitopes. This conclusion is supported by the observation that a neutralising response is enhanced when both outer capsid proteins are present as antigens, in comparison to VP2 on its own (MartinezTorrecuadrada *et al.*, 1996).

3.3.2: The core proteins: VP1, VP3, VP4, VP6 and VP7

VP3 and VP7: Both, the core surface protein VP7 and the sub-core shell protein VP3 of AHSV are highly conserved with a minimum nt identity >89.1% and >95.5% (in Seg-8 and Seg-3), or aa identities of >98% and >99.2% respectively, for the reference strains analysed here (Figure 3.47). In fact VP3 is the most conserved AHSV protein among the strains tested (Figure 3.37). The high level of VP3 and VP7 conservation reflects the functional and structural importance of these proteins.

In comparison, the overall nt and aa conservation within the two major BTV core proteins was significantly lower at: >65.4% / >53.1% for Seg-7 / VP7, and >74.9% / >87.8% for Seg-3 / VP3 (Maan *et al.*, 2010b). The values for EHDV (>77.9% / >90.3% for Seg7 /

VP7, and >79.3% / >95.5% for Seg-3 / VP3) (Anthony *et al.*, 2009a) are also much lower than in AHSV reflecting, once more, their larger geographical distribution (topotypes), and the analyses of more BTV and EHDV isolates, showing that most of their genes including Seg-3/VP3 exist as distinct eastern and western topotypes (Anthony *et al.*, 2009a; Maan *et al.*, 2010b; Nomikou *et al.*, 2009). However, within the same topotype, Seg-3/VP3 of BTV and EHDV show similar levels of sequence identity to those described for AHSV.

Despite the fact the both VP3 and VP7 proteins of AHSV are under very strong purifying selection (d_N/d_S=0.012 and d_N/d_S=0.017 respectively) and their nucleotide substitution rates are in the lower range (Table 3.22), the Seg-8 nucleotide sequence is about 10% more variable than the amino acid sequence. Similar observations have been described in BTV and EHDV (Anthony *et al.*, 2009a; Maan *et al.*, 2010b) suggesting that there is a scope for some nucleotide variation.

The core surface layer is organised in so that VP7 interacts individually with VP2, VP5 and VP3 (Grimes *et al.*, 1995; Nason *et al.*, 2004; Stuart *et al.*, 1998). However, these close structural interactions between the proteins are not reflected in their phylogenetic groupings (Figures 3.10, 3.11, 3.14, 3.15, 3.27, 3.28, 3.32 and 3.33). In ML based phylogenetic analysis of VP7 and VP3, nucleotide variations between the individual strains of AHSV analysed, were reduced to only few versions of the proteins, shared between the strains. This indicates that, despite structural interaction between the proteins, the serotype related grouping of VP2 and VP5 does not affect VP7 phylogeny. The grouping of isolates, based on VP7 also does not resemble that of VP3, indicating independent functions of these proteins and independent reassortment of Seg-3 and Seg-8. The fact that only 5 different closely related versions of VP7 (Figure 3.38) and 4 of VP3 (Figure 3.15) are used by the nine reference strains of AHSV points to the

importance for the virus to have stable genetics of these proteins to maintain its structural integrity and proper interactions between remaining structural proteins. It also suggests that genome segment reassortment and environmental selection has led to a restricted library of these particular genes.

Phylogenetic analysis of VP7 from BTV and EHDV, grouped isolates into clusters that are only partially dependent on geographical origin but independent of serotype (Anthony *et al.*, 2009a; Maan *et al.*, 2010b). As BTV core particles were shown to have a high 'specific infectivity' for insect cells, it was suggested that these clades may relate to the different insect vector species/populations that transmit the virus (Mertens *et al.*, 1987; Mertens *et al.*, 1996; Xu *et al.*, 1997). Even if major regional topotypes are not found, similar different and potentially functional clades could still exist for Seg-8/VP7 of AHSV. Further studies, involving different AHSV insect vectors, more virus isolates, and/or reverse genetics studies would needed to test this hypothesis.

In BTV and EHDV, the sub-core proteins (VP1, VP3, VP4 and VP6) all group accordingly to their geographical origin into eastern and western topotypes (Anthony *et al.*, 2009a; Maan *et al.*, 2008; Maan *et al.*, 2010b; Nomikou *et al.*, 2009) as a result of independent, long term genetic drift in non-overlapping geographical areas (Bonneau *et al.*, 2001; Pybus & Shapiro, 2009). Topotypes were not identified in any of the AHSV genome segments and have not been described in the literature. This is, most probably related to the fact that AHSV only exists in a confined and contiguous geographical area, where different lineages of the virus are not sufficiently separated to have diversified into distinctive topotypes.

VP1, VP4 and VP6: VP1, VP4 and VP6 make up the transcriptase complexes, which carry out AHSV RNA transcription and replication of within the core particle structure

(Mertens & Diprose, 2004). Each of the transcriptase complex' protein sequences and their respective genome segments are highly conserved (Figure 3.47) with similar values of minimum nt and aa identities to those of BTV and EHDV within a single topotype (Anthony *et al.*, 2009a; Maan *et al.*, 2010b). This suggests that the orbiviruses are able to 'accept' only a limited amount of variation within their replication machinery to maintain its function. Changes in both VP1 and VP4 are under very strong purifying/negative selection (d_N/d_S=0.029 and d_N/d_S=0.067, respectively) and they appear to be evolving at a relatively low rate (8.16 x 10^{-2} and 6 x 10^{-2} nt substitutions per site, respectively). In contrast VP6 evolves at a much faster rate (2.1 x 10^{-1} nt substitutions per site) compared to any of the other AHSV proteins, (with much less negative selection and almost neutral pressure on the protein coding sequence (d_N/d_S=0.883) Table 3.22). Interestingly, the estimated d_N/d_S ratio for VP6 of BTV is significantly lower value (d_N/d_S=0.242) (Maan *et al.*, 2011b), similar to these of VP2 and NS3 of AHSV (Table 3.22). The low negative pressure on AHSV VP6 may represent an AHSV specific feature of the protein. Alternatively this value may be influenced by the small number of VP6 coding sequences that were used for these calculations. The nine AHSV reference strains were highly passaged in mammalian cells, and the natural selection on this particular protein could have been compromised, by the lack of alternative passages in insect vector cells. To ensure a true estimate of the selection pressure on VP6, more analyses need to be performed on a larger number of low passaged viruses.

3.3.3: The non-structural proteins: NS1, NS2, NS3 and NS4

Apart from the structural proteins, AHSV genome encodes four distinct non-structural proteins: NS1, NS2, NS3 / NS3a and NS4, encoded by genome segments 5, 9, 10 and 7

respectively (Belhouchet *et al.*, 2011; Bremer *et al.*, 1990; O'Hara *et al.*, 1998; Ratinier *et al.*, 2011). Non-structural proteins do not take part in the composition of the virus particle but are only found in infected cells where they are involved in virus replication and assembly or interactions with the host cells / systems, in a manner that may influence pathogenesis and morphogenesis (NS1) (Owens *et al.*, 2004), replication and packaging (NS2) (Kar *et al.*, 2007), intracellular trafficking and release of virus particle (NS3/ NS3a) (Celma & Roy, 2009; Martin *et al.*, 1998; Stoltz *et al.*, 1996) and counteraction of antiviral responses of the host (NS4) (Ratinier *et al.*, 2011). Most of the AHSV genome segments encode a single protein (Bremer *et al.*, 1990; O'Hara *et al.*, 1998), apart from Seg-10 which encodes two non-structural proteins: NS3 and NS3a (Bremer *et al.*, 1990; O'Hara *et al.*, 1998) and Seg-6 which codes a structural protein (VP6) and a non-structural protein (NS4) from overlapping open reading frames (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011).

NS1 and NS2: AHSV NS1 and NS2 proteins are both very highly conserved, with very close values for their minimum nt and aa sequence identities within the group of reference viruses analysed (95.6%/98% for Seg-5/NS1: 96.7%/98.1% for Seg-9/NS2 - Figure 3.47). Interestingly, the two structure controlling proteins, VP7 and VP3 are also conserved at comparable level. NS1 and NS2 also evolve at similar rate (3.3×10^{-2} and 2.5×10^{-2} nucleotide substitution per site respectively) and both of the proteins are under strong negative purifying selection (NS1: d_N/d_S=0.067 and NS2: d_N/d_S=0.052; Table 3.22).

The phylogenetic groupings of NS1 and NS2 confirm that the isolates examined are closely related but their tree topologies appear to be different from each other (Figure 3.22, 3.23, 3.35 and 3.36) and from those of the other genome segments and proteins of AHSV.

The core proteins and non-structural proteins NS1 and NS2 of BTV and EHDV, display clear east-west topotype grouping, which are not evident in AHSV (Anthony *et al.*, 2009b; Maan *et al.*, 2010b). These BTV and EHDV topotypes are related to the overall geographical distribution of the viruses (across several separated continents), the origins of individual isolates and they contributed to the overall genetic variation detected within each genome segment and the proteins they encode. NS1 and NS2 of BTV and EHDV show much higher overall levels of variation than those of AHSV, which is endemic only in Africa. However, genetic variation within most of the individual topotypes of BTV and EHDV also exceeds that detected in AHSV, although this may reflect the small number of virus strains analysed here (Anthony *et al.*, 2009b; Maan *et al.*, 2010b).

NS3: Unlike the very conserved NS1 and NS2 proteins, NS3 is the second most variable AHSV protein (after VP2) with minimum nt and aa identity levels of 67.9% and 66% respectively and groups as three different clades: α, β and γ (Figure 3.45 3.46 and 3.47) (Martin *et al.*, 1998; Quan *et al.*, 2008; Sailleau *et al.*, 1997b; van Niekerk *et al.*, 2003). Although Seg-10/NS3 of BTV and EHDV group in 'semi-geographic' manner and also exist as more than one cluster in each topotype (Anthony *et al.*, 2009b; Maan *et al.*, 2010b), despite their greater geographical distribution, they are significantly less variable overall than those of AHSV (BTV: 75.9%/81.2%, EHDV: 74.6%/83.8%), (Anthony *et al.*, 2009b; Maan *et al.*, 2010b; van Niekerk *et al.*, 2003).

In this study, some of the Seg-10/NS3 clusters partially reflected the serotype specific grouping of VP2 (Figure 3.10 and 3.11) with isolates RSArrah/01 and RSarrah/02 (cluster γ), RSArrah/03 and KENrrah/07 (cluster β) grouping together but separate from other serotypes. Cluster α includes the five remaining isolates. A recent study investigating partial sequences of multiple strains of different AHSV serotypes (Quan *et al.*, 2008) concluded that isolates of certain serotypes always grouped in specific Seg-10/NS3

clusters (AHSV serotypes 6, 8 and 9 in cluster α; serotypes 3 and 7 in β; and serotype 2 in γ). The others (serotypes 1, 4 and 5) were found to group in clusters α and γ. This suggests that NS3/NS3a can interact in some way with outer capsid proteins VP2 (possibly during cell exit) (Beaton *et al.*, 2002).

Studies of AHSV virulence in mice (an animal model for AHSV) using reassortant viruses, concluded that genome segments 2, 6 and 10, coding for VP2, VP5 and NS3/NS3a respectively, could play a role in determining virulence of the virus (O'Hara *et al.*, 1998). These proteins are involved in virus entry (VP2 and VP5) (Forzan *et al.*, 2004) or release (NS3/NS3a) (Celma & Roy, 2009; Martin *et al.*, 1998; Stoltz *et al.*, 1996) and are therefore involved in processes that are vital for infection, pathogenesis and transmission of the virus. Similarities in the phylogenetic grouping of isolates, for these genome segments (Figures 3.10, 3.11, 3.27, 3.28, 3.45 and 3.46), suggests a need for further studies to investigate interactions and the roles of VP2, VP5 and NS3/NS3a in AHSV virulence, possibly by using reverse genetics techniques to make specific changes / reassortants.

Estimation of nucleotide substitution rates in the NS3 OFR indicate that this protein evolves at fast rate of 1.16 nucleotide substitutions per site; comparable to VP2 (1.96 nucleotide substitutions per site) (Table 3.22). These two segments are the fastest evolving of the whole AHSV genome. Despite the fast evolution, NS3 is still under overall negative selective pressure, with a d_N/d_S value of 0.222, in line with VP2 (d_N/d_S=0.317) (Table 3.22). Selective pressure for the NS3 gene in BTV, is lower (d_N/d_S=0.060) (Carpi *et al.*, 2010)/(d_N/d_S=0.047) (Maan *et al.*, 2010b) indicating that NS3/NS3a of BTV (and possibly EHDV) are under different genetic constrains from those of AHSV.

NS4: Recent bioinformatics studies identified an additional orbivirus protein encoded by a 2nd downstream +1 ORF, overlapping with the VP6 ORF on Seg-7 (Firth, 2008). NS4 expression was subsequently demonstrated in infected mammalian and insect cells (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011). In all nine historical strains of AHSV the translation of NS4 starts from an initiation codon at 193 bp and may terminate at 657 bp (or position 651 for RSArrah/08 due to 6-nucleotide deletion within the ORF) (Table 3.13). However the majority consensus sequence of RSArrah/02, RSArrah/05 and PAKrrah/09 indicates another premature termination of the protein due to a single nucleotide substitution resulting in a formation of an earlier stop codon at positions of 579, 600 and 534 bp respectively, potentially reducing the protein from 154 aa to 128, 135, and 113 aa respectively. It has been shown that NS4 is dispensable in the *in vitro* replication of BTV in both insect and mammalian cell cultures (Ratinier *et al.*, 2011) and, therefore, as described for NS3 of BTV (Bonneau *et al.*, 2001), a random substitution leading to the creation of a premature stop codon, could have been a result of genetic drift. However NS4 may be immportant *in vivo* to combat the hosts' antiviral resposes. Uncorrected, by alternated replication of the virus in mammalian and vector species, these early stop codons could have become fixed through multiple page in BHK cell culture.

Careful examination of sequencing chromatograms at the respective stop codons of RSArrah/02, RSArrah/05 and PAKrrah/09 revealed a presence of a mixed nucleotide at the relevant position, suggesting that the virus contained a mixed population expressing both truncated (major component) and full-length NS4 (minor component). The coexistence and balance / ration of different versions of a genome segment could potentially change during AHSV infection of the host and vector, as speculated for BTV (Bonneau *et al.*, 2001) and AHSV (Manole *et al.*, 2012; Potgieter *et al.*, 2009).

The overall substitution rate in the NS4 and VP6 ORF (2.8×10^{-2} and 2.1×10^{-2} nucleotides substitutions per site, respectively) is relatively low in comparison to other genes of AHSV (Table 3.22). This may be, at least, partially explained by the overlapping nature of the VP6 and NS4 ORFs, leading to two sets of conservation pressures, and in effect, the absence of a more flexible 'third base' position. Interestingly, NS4 is under overall negative selective pressure (d_N/d_S=0.289) at a comparable level to that of VP2 (d_N/d_S=0.317), which is greater than VP6 (d_N/d_S=0.883). This could be explained by the fact that NS4 is not essential for replication in either mammalian or insect cells (Ratinier *et al.*, 2011), allowing it considerable freedom to change its aa sequence. There is no available data on substitution rate or selective pressure on NS4 encoded by other orbiviruses.

Phylogenetic analysis of NS4 sequences showed similar grouping of isolates / serotypes as in VP6 (Figure 3.33 and 3.34), which appears likely to reflect the inevitable co-segregation of the two proteins during reassortment. Like in VP6, there is no evidence for topotypes in NS4 but, so far, there are no data concerning phylogenetic grouping of NS4 form other orbiviruses, for comparison.

3.3.4: Terminal hexonucleotides

A common feature in the genus *Orbivirus* and in other reoviruses is high level of conservation in the 5' and 3' terminal sequences of the viral RNAs (Mertens *et al.*, 2005) (http://www.reoviridae.org/dsRNA_virus_proteins/CPV-RNA-Termin.htm). These sequences are also highly conserved within the nine historical reference strains of AHSV; both 3' and 5' terminal hexanucleotides were identical in four of the 'conserved' genome segments (Seg-1, 3, 5 and 7) (Table 3.23). The 3' terminal hexanucleotide was also fully

conserved in Seg-9 and Seg-10. The consensus terminal hexanucleotide sequences for all ten genome segments of the nine reference strains, can be expressed as 5'-GTTWAW.....MCHTAC-3' (W = T or A, M = A or C, H = T, A or C).

Table 3.23: Consensus sequences of AHSV terminal hexanucleotides.

Genome segment	5' termnal hexanucleotides	3' terminal hexanucleotides
Seg-1	5'-GTTTAT...	...ACTTAC-3'
Seg-2	5'-GTTWAW...	...MCWTAC-3'
Seg-3	5'-GTTTAT...	...ACTTAC-3'
Seg-4	5'-GTTWAT...	...CCTTAC-3'
Seg-5	5'-GTTAAA...	...ACTTAC-3'
Seg-6	5'-GTTWAT...	...ACWTAC-3'
Seg-7	5'-GTTAAA...	...ACTTAC-3'
Seg-8	5'-GTTWAT...	...ACYTAC-3'
Seg-9	5'-GTTWAT...	...ACATAC-3'
Seg-10	5'-GTTWAA...	...ACTTAC-3'

Differences between the sequence data from this study and the data published in VIIIth Report of the ICTV (Mertens *et al.*, 2005) are highlighted in dark blue. W = T or A, M = A or C, Y = C or T.

While the 5' terminal hexanucleotide of the AHSV strains investigated are similar to those published for other *Orbiviruses* (Anthony *et al.*, 2009a, b; Anthony *et al.*, 2009c; Attoui *et al.*, 2009a; Maan *et al.*, 2010b), there are some differences in the 3' hexanucleotide compared to previously published data (Table 3.23). The consensus 3' terminal hexanucleotide is different from previously published reports for the consensus terminal sequences of the full genome of AHSV-1 and -2 which can be expressed as ... YTHUAC-3' (Potgieter *et al.*, 2002). This may reflect the analysis of different or more isolates, or the high passage levels of some strains in cell culture. These conserved terminal regions are thought to be important as recognition signals for the initiation of

transcription and RNA packaging (Rao *et al.*, 1983) and it has not been shown what impact differences in these regions might have.

In BTV and EHDV, the downstream terminal hexanucleotides are also reported to be less conserved than the upstream sequence (Anthony *et al.*, 2009a, b; Anthony *et al.*, 2009c; Maan *et al.*, 2010b). This may indicate that, in general, there is a higher pressure to conserve 5' terminal sequences in orbiviruses, over the 3' hexanucleotides.

3.3.5: Selective pressure on AHSV genes

Viruses, which for successful transmission requires alternate passage in mammalian and insect cells, such as the RNA arboviruses, have been reported to be under increased selective constrains (Coffey *et al.*, 2008; Greene *et al.*, 2005; Jenkins *et al.*, 2002; Weaver *et al.*, 1999). All eleven protein coding sequences of the nine AHSV reference strains, are under a general negative pressure ($d_N/d_S<1$) (Table 3.22) but with different d_N/d_S values in their different genes. Estimates of selection pressures for each genome segment were matched with inferred nucleotide substitution rates, to better understand the evolutionary dynamics and their effect on the biological function of each coding sequence. Thus, VP7 and VP3, proteins, which act as the scaffolding of the virus particle structure (Grimes *et al.*, 1995; Stuart *et al.*, 1998) are under strictest general purifying force (d_N/d_S of 0.017 and 0.012 respectively). Coupled with their low nucleotide substitution rate (3.68×10^{-2} and 5.6×10^{-2} substitutions per site respectively) (Table 3.22) this indicates the high relative importance of preserving the individual protein conformation and therefore the structure of the virus particle itself, for successful transmission and replication.

In contrast, less negative pressure is applied to VP2 and NS3, the most variable of the AHSV proteins, with d_N/d_S values closer to 1 (at 0.317 and 0.222 respectively). VP2 and

NS3 also have the most frequent rate of nucleotide substitutions (1.96 and 1.16 substitutions per site respectively) (Table 3.22). These findings are easily explained for VP2, due to its outer-capsid location and its role as a serotype determinant (Manole *et al.*, 2012; Roy *et al.*, 1994; Zhang *et al.*, 2010). VP2 responds to the host's immunological pressures, by more frequent nt and aa changes than any of the AHSV genes / proteins.

However, the reason for a reduced selection pressure on NS3 and its relatively faster evolution are less clear. It may reflect the involvement of NS3 in cell exit, which may be particularly important in dissemination in different insect vector species. Alternatively NS3 has been implicated in control of AHSV virulence (Huismans *et al.*, 2004; O'Hara *et al.*, 1998). However, VP5 was also found to contribute to virulence in mice model (O'Hara *et al.*, 1998) and is present in the outer capsid, but only evolves at intermediate rate (3.4×10^{-1} substitutions per site) under strong purifying selection (Table 3.22).

Surprisingly, the highest overall d_N/d_S value of 0.883 was found for gene coding for VP6 (Table 3.22), even though it is a highly conserved protein with the viral helicase activity (Mertens & Diprose, 2004). Instinctively, VP6 function should be protected by negative purifying selection pressure, in line with other proteins making up the AHSV transcriptase complex (VP1 and VP4), in order to maintain efficient replication of the virus. However the evolutionary pressure on the other enzymatic core-proteins is in a different range (Table 3.22). The OFR of VP6 overlaps with NS4 ORF (which has a d_N/d_S =0.289) which, in theory, reduces the chance of deletion mutations in either of the genes and should magnify the purifying selection on both of the genes. However, analysis of the NS4 coding sequence showed introduction of premature stop codons within three different AHSV strains (section 3.2.3.9). This suggests that the negative selection on both proteins could be less stringent.

Previous studies have showed that multiple passages of arboviruses in a single host cells led to an increase in mutation numbers (Coffey *et al.*, 2008; Greene *et al.*, 2005; Weaver *et al.*, 1999). Possibly, in AHSV, this phenomenon is reflected by increased random mutations (genetic drift) within Seg-7.

As relatively small number of AHSV isolates, which had been repeatedly passaged in mammalian cells, was used in this study, the evolutionary rates of the AHSV genes were only estimated as nucleotide changes per site, rather than over a specific time period. Carpi *et al.*, 2010, in their study on BTV, used a large number of sequences per gene from well documented samples collected over an extended period, which allowed them to estimate evolutionary dynamics by a Bayesian coalescent method and therefore calculated the substitution rates as nucleotide substitutions per site per year. These differences in experimental approach make comparisons of the substitution rates in AHSV and BTV genes (based on published data) difficult.

Overall, despite being under general negative pressure, the different genes of AHSV appear to evolve in relation to the function of their coded proteins, but independently from each other. These findings are in agreement with previous studies on BTV, which also show negative selection and independent evolution of respective genes, reflecting the ability of these viruses to reassort genome segments (Carpi *et al.*, 2010; Maan *et al.*, 2011b; Maan *et al.*, 2010b; Nomikou *et al.*, 2009). It will be valuable to extend the database for AHSV to include whole genome sequence data for additional field isolates from different locations, dates and host species. This will increase its value for molecular epidemiology studies, and help to verify / test the findings concerning gene and virus strain evolution described here.

Chapter 4:
Real time RT-PCR assays for detection and typing of AHSV

4.1: Introduction

AHS is a rapid, lethal disease of horses, which is currently restricted to sub-Saharan Africa. In non-endemic areas, the disease which is transmitted by *Culicoides* vectors, can spread very rapidly and persist over extended period of time (Chapter 1 section 1.7.1) causing extensive damage to equine industries (Chapter 1, section 1.7.6). Therefore there is a need for accurate and rapid diagnostic systems for AHS, for: (a) identification of AHSV in clinical samples, isolates and outbreaks; (b) surveillance programmes; (c) demonstration of an absence of the infectious agent in individual animals and animal products (for safe movements); (d) demonstration of a 'virus free' state in specific countries or zones (OIE, 2011).

Currently there are number of different diagnostic methods for AHSV (Chapter 1, section 1.8) including detection the virus by real-time RT-PCR. This method offers many advantages to researchers and diagnosticians, including: (a) detection of a short amplicon, reducing potential problems related to target degradation, which may be especially important for field samples; (b) the RT-PCR reactions and detection of the product takes place in a close tube, which reduces the chances of laboratory based cross-contamination, reducing the chances of false positive results; (c) automation of the assay and detection of the product, increases throughput; (d) real-time RT-PCR are highly sensitive and specific; (e) real-time RT-PCR are suitable for quantitation of viral RNA templates (copy number).

However, the very high specificity of real-time RT-PCR primers and probes can also lead to a failure of the method to detect positive samples if they have even relatively few

mismatches (Jiménez-Clavero *et al.*, 2006). This suggests that for the design of real-time RT-PCR the most conserved genome segments and regions should be selected and a large selection of samples, including field and historical isolates should be used for assay validation.

Despite rapid progress in the development of methods for identification of the different AHSV serotypes in recent years (Chapter 1, section 1.8), neither of the currently available methods (Virus Neutralisation Tests (VNT) and conventional RT-PCR methods targeting VP2) can be reliably used in a high throughput format.

The live attenuated AHSV vaccine strains that are currently available (from Onderstepoort Biological Products (OBP), South Africa) generate serotype specific protection (Coetzer & Guthrie, 2004). Although these strains are currently used in AHSV endemic areas, as part of polyvalent vaccine preparations, their use risks introduction of additional AHSV serotypes into the vaccinated region. Rapid determination of AHSV type, especially for outbreaks in non-endemic areas, would facilitate the implementation of a more 'precise' vaccination campaign to help control the disease and virus spread, using only a single vaccine 'type' that specifically targets the outbreak strain (Chapter 1, section 1.9).

This chapter describes the development and evaluation of a set of real-time RT-PCT assays for detection and typing of AHSV. Two independent group-specific assays, each of which detects all nine serotypes of AHSV, were designed to amplify specific regions of Seg-1 or Seg-3 respectively. A set of nine individual real-time RT-PCR assays were also designed targeting Seg-2 of each AHSV serotypes. Assay specificity was evaluated using a wide range of AHSV isolates from the Orbivirus Reference Collection (ORC), including reference, vaccine and field strains (Mertens & Attoui, 2011).

4.2: Development of AHSV specific and typing real-time RT-PCR assays

4.2.1: Virus isolates

A panel of 42 AHSV isolates, representing all nine of the known serotypes, including monotypic reference, field and vaccine strains from different geographical locations, were used for the development and evaluation of real-time RT-PCR assays (Table 4.1). The isolates used in the study were obtained from the ORC at the Pirbright Institute (Mertens & Attoui, 2011). All field isolates were obtained from diagnostic samples, including blood and/or tissues (spleen or lungs) of naturally infected animals and were taken as a part of normal diagnostic investigations carried out by qualified veterinarians in the countries of origin.

4.2.2: Primers and probes design

AHSV Seg-1, Seg-2 and Seg-3 nucleotide sequences were generated during this study, or were obtained from the public domain (NCBI), for isolates including all nine AHSV serotypes (Table 2.5). Sequences of different genome segments were aligned and analysed using CusterW software (Thompson *et al.*, 1994). Conserved regions in Seg-1 and Seg-3 were identified across for all nine serotypes, and primers and probes were designed for two separate assays in accordance with TaqMan specifications (Chapter 2, section 2.8).

Genome segment sequences 2 were also analysed collectively and separately for each of the nine AHSV serotypes. Unique regions were identified as targets for primers and probes, for each of type.

Table 4.1: AHSV isolates used to evaluate real time RT-PCR assays for AHSV.

Virus serotype	Origin	ORC reference number [2]
Reference strains [1]		
AHSV-1	Republic of South Africa	RSArah1/03*
AHSV-2	Republic of South Africa	RSArah2/03*
AHSV-3	Republic of South Africa	RSArah3/03*
AHSV-4	Spain	SPArah4/03*
AHSV-5	Republic of South Africa	RSArah5/03*
AHSV-6	Republic of South Africa	RSArah6/03*
AHSV-7	Kenya	KENrah7/03*
AHSV-8	Republic of South Africa	RSArah8/03*
AHSV-9	Pakistan	PAKrah9/03*
Field strains		
AHSV-2	Ethiopia	ETH2010/01*
AHSV-2	Ethiopia	ETH2010/20*
AHSV-2	Ghana	GHA2010/01*
AHSV-2	Ghana	GHA2010/02*
AHSV-2	Ghana	GHA2010/03
AHSV-2	Ghana	GHA2010/04
AHSV-2	Ghana	GHA2010/05
AHSV-2	Senegal	SEN2007/01
AHSV-2	Senegal	SEN2007/02*
AHSV-2	Senegal	SEN2007/03
AHSV-2	Senegal	SEN2007/04*
AHSV-2	Senegal	SEN2007/05*
AHSV-4	Ethiopia	ETH2010/03
AHSV-4	Ethiopia	ETH2010/04
AHSV-4	Ethiopia	ETH2010/05*
AHSV-4	Ethiopia	ETH2010/06
AHSV-4	Ethiopia	ETH2010/07*
AHSV-4	Kenya	KEN2007/01*
AHSV-4	Spain	SPA1987/01*
AHSV-6	Ethiopia	ETH2010/19*
AHSV-7	Senegal	SEN2007/06*
AHSV-8	Ethiopia	ETH2010/10*
AHSV-8	Ethiopia	ETH2010/11*
AHSV-9	Ethiopia	ETH2010/14
AHSV-9	Ethiopia	ETH2010/15
AHSV-9	Ethiopia	ETH2010/16*
AHSV-9	Gambia	GAM2009/01*
AHSV-9	Gambia	GAM2009/02*
AHSV-9	Kenya	KEN2006/01*
Vaccine strains		
AHSV-2+3	Ethiopia	ETH2010/02*
AHSV-5+7	Ethiopia	ETH2010/09*
AHSV-5+9	Ethiopia	ETH2010/18
AHSV-9	Senegal	SENvvv1/09*

Further details on these isolates can be accessed through http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm (Mertens & Attoui, 2011). [1] Set of nine monotypic AHSV reference strains (section 4.2.4.2); *Seg-2 sequenced.

Table 4.2: List of primers and probes for AHSV group and type specific assays.

Assay Type	Oligo Name	Oligo Sequence (5'-3')
Seg-1 based/Group-specific		
	AHSV/Seg-1/FP/1310-1329	CCTGAAGATATGTATACATC
	AHSV/Seg-1/RP/1444-1461	ACTAACGCTTTAATCCGC
	AHSV/Seg-1/P/1400-1423	CCCAACGCRAARGGTGGACGTGG
Seg-3 based/Group-specific		
	AHSV/Seg-3/FP/2030-2050	AGATATYGTAAGGTGGAGTCA
	AHSV/Seg-3/RP/2115-2136	CTAACATCAARTCTTCAAARTC
	AHSV/Seg-3/P/2085-2107	ATCGCCCAAGCTTCCCTATTCAA[#]
Seg-2 based/Type-specific		
	AHSV/SRT-1/FP/1143-1163	GAATGGTAAGCTTTGGATTGA
	AHSV/SRT-1/RP/1225-1207	TTAGAGGCGCTCGGTTCTC
	AHSV/SRT-1/P/1165-1187	CATAAACAAACGGTGAGTGAGCA
	AHSV/SRT-2/FP/1763-1783	AGTGGACTTCGATTATAGATG
	AHSV/SRT-2/RP/1897-1878	CTGTCTGAGCGTTAACCTTC
	AHSV/SRT-2/P/1863-1842	TTCAACCGTCTCTCCGCCTCTC[#]
	AHSV/SRT-3/FP/809-829	TAGAAAGAATGATGAGCAGTG
	AHSV/SRT-3/RP/901-921	TAATGGAATGTCGCCTGTCTT
	AHSV/SRT-3/P/845-870	CCGTTATTGAGAGCGTCATAAGATTC
	AHSV/SRT-4/FP/1934-1956	GTTTCGTATCATATTGGTATAGA
	AHSV/SRT-4/RP/2079-2101	GATATATATATGTGGATGCATGG
	AHSV/SRT-4/P/1996-2020	CYGGAYATGAATGAGAAACAGAAGC
	AHSV/SRT-5/FP/1875-1895	GAGACACATCAAGGTTAAAGG
	AHSV/SRT-5/RP/2035-2014	CAGGATCAAACTGTGTATACTT
	AHSV/SRT-5/P/1964-1985	TTGAAGCAAGGAATCTATTGACTTT
	AHSV/SRT-6/FP/1285-1304	GTTGGTCATTGGGTCGATTG
	AHSV/SRT-6/RP/1505-1485	GACGAATTTGATCCCTTCTTG
	AHSV/SRT-6/P/1385-1408	TGATGTCGGGTATGAACAAACTGG
	AHSV/SRT-7/FP/1159-1179	TGGATCGAGCATAAGAAGAAG
	AHSV/SRT-7/RP/1282-1302	CCAATCAACCCARTGTGTAAC
	AHSV/SRT-7/P/1213-1237	ACCAAAATCGTCCGATGCTAGTGC
	AHSV/SRT-8/FP/2122-2141	GGTGAGCGGATTGTTGATAG
	AHSV/SRT-8T/RP/2293-2274	CTGAATCCACTCTATACCTC
	AHSV/SRT-8/P/2169-2146	CGACAAATCATACCACACGATCTC[#]
	AHSV/SRT-9/FP/ 1931-1952	TATCATATTGGTATCGAGTTCG
	AHSV/SRT-9/RP/2074-2054	AAGTTGATGCGTGAATACCGA
	AHSV/SRT-9/P/1978-2000	ACATCCTCAATCGAYCTCCTCTC[#]

The names of the oligos indicate the type of assay (Seg-1, Seg-3 indicate respective group specific assay, while SRT-1 to SRT-9 stand for respective typing assays), FP, RP, P stand for forward primer, reverse primer and probe respectively and numbers represent annealing positions on the genome segment. Probes indicated by an asterisk (*) are complementary to the negative strand.

The sequences of different primers and probes (Table 4.2) were analysed *in silico* to ensure no cross-reactions with closely related orbiviruses. The probes were labelled at their 3' and 5' ends with BHQ-1 (Black Hole Quencher-1) and FAM respectively. All oligonucleotides were manufactured by Eurogentec.

4.2.3: Optimisation of the assays conditions

In order to achieve low cycle threshold (Ct) values and high fluorescence signals, the individual steps of the method were optimised separately for each of the assays, including: (a) primers concentrations, (b) primers-probe ratios, (c) magnesium concentration, (d) reverse transcription temperature, (e) reverse transcription period, (f) annealing temperature, and (g) annealing time (data not shown).

The optimal concentration of each primer in individual assays was evaluated in chequerboard experiments, where combinations of different primers concentrations were tested for each of the assays. As probes were not included in these assays, template amplification was detected in SYBR Green (Sigma) reactions. To determine optimal primer-probe ratio for each of the assays, the optimal primers concentrations were tested with staggered concentrations of probe in TaqMan assays. Optimisation of assay composition was achieved by testing different magnesium sulphate ($MgSO_4$) concentrations. The different primers-probe sets were used to amplify a fragment of the targeted genome segment, using SuperScript III/ Platinum Taq One-Step qRT-PCR Kit (Invitrogen). The composition of the individual assays, after optimisation, is presented in Table 4.3.

dsRNA samples were heat denaturated for 5 min at 95°C (Shaw *et al.*, 2007) prior to addition to the reaction mix. To avoid accidental contamination, assembly of the reaction

mix was conducted in a laminar flow hood dedicated to 'clean work', which is separate to the RT-PCR assembly hood. Amplification for all of the assays was carried out in MX3005p (Stratagene) and was optimised for the same amplification conditions : 55°C for 30 min, 95°C for 10 min followed by 45 cycles of 95°C for 30 s and 55°C for 1 min. Fluorescence was measured at the end of the 55°C annealing/extension step. Cycle threshold (Ct) values were measured at the point at which the sample fluorescence signal crossed a threshold value (the background level). Negative results (for assays that did not exceed this level of signal) are reported as 'No Ct'.

Table 4.3: RT-PCR assays composition.

Reagent	**Assay detecting segment**		
	Seg-1	**Seg-3**	**Seg-2**
Forward primer (10µM)(µl)	2	1	2
Reverse primer (10µM)(µl)	2	1	2
Probe (5µM)(µl)	1.5	1	0.5
MgSO4*(µl)	0.5	1.5	1
SuperScript III RT/Platinum *Taq* Mix*(µl)	0.5	0.5	0.5
2x Reaction Mix*(µl)	12.5	12.5	10
Nuclease free water (µl)		1.5	
dsRNA(µl)	6	6	4
total volume(µl)	**25**	**25**	**20**

Composition of each of the RT-PCR assays was optimised to achieve low Ct values and high fluorescence. Reagents marked with an asterisk (*) are part of the SuperScript III/ Platinum Taq One-Step qRT-PCR Kit (Invitrogen).

4.2.4: Diagnostic sensitivity and specificity

Diagnostic sensitivity refers to the ability of an assay to detect a specific pathogen. Assays of high diagnostic sensitivity should be able to detect a wide range of samples of the target pathogen from different geographical origins and, in case of group specific assays, all of its different serotypes.

The diagnostic specificity of the assay is defined as its ability to detect only the target sequence of a pathogen or its types. Specific assays offer accurate diagnosis through avoidance of false positive results, caused by cross reactions with even closely related but distinct pathogens.

4.2.4.1: Seg-1 and Seg-3 based (serogroup) assays

A panel of nine monotypic AHSV reference strains, representing the nine known serotypes of AHSV (Table 4.1) were tested in duplicate, with both of the group-specific assays, targeting Seg-1 or Seg-3 respectively. The resulting mean Ct values for most of the strains tested in the Seg-1 assay were similar to those obtained in Seg-3 based assay, indicating that both of the tests can detect viral RNA with similar efficiency (Table 4.4). However, RNA of AHSV-9 (PAHrah9/03), was detected 4 Ct values earlier with the Seg-3 assay, in comparison to the Seg-1 assay (Table 4.4).

The diagnostic specificity of the AHSV group-specific assays was further evaluated using 33 different field and vaccine strains of AHSV serotypes 2, 3, 4, 6, 7, 8, and 9, from different geographical location in central Africa (Table 4.1). All of the isolates were detected (produced amplification) in both assays (Table 4.4). Most of the isolates tested (17 out of 33) were detected with similar sensitivity (within 2 Ct value difference). While the Seg-1 assay amplified the RNA target earlier (lower Ct) in thirteen isolates. The Seg-3 based assay was more efficient in amplification (lower Ct) with RNA samples from the three Ethiopian isolates of AHSV-4 (Table 4.4).

Table 4.4: Diagnostic sensitivity of group (Seg-1 and 3) and type specific (Seg-2) assays.

AHSV isolate	Group specific		AHSV Type								
	Seg-1	Seg-3	-1	-2	-3	-4	-5	-6	-7	-8	-9
Reference											
RSArah1/03	17.36	19.13	18.39	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
RSArah2/03	16.56	16.02	No Ct	15.57	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
RSArah3/03	21.1	22.02	No Ct	No Ct	21.18	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
RSArah4/03	14.37	16.09	No Ct	No Ct	No Ct	17.1	No Ct	No Ct	No Ct	No Ct	No Ct
RSArah5/03	19.02	20.52	No Ct	No Ct	No Ct	No Ct	22.67	No Ct	No Ct	No Ct	No Ct
RSArah6/03	20	19.81	No Ct	No Ct	No Ct	No Ct	No Ct	22.95	No Ct	No Ct	No Ct
KENrah7/03	17.22	16.17	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	19.24	No Ct	No Ct
RSArah8/03	17.08	16.41	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	18.41	No Ct
PAKrah9/03	16.73	12.63	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	11.73
Field strains											
ETH2010/01	18.41	17.44	No Ct	15.51	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
ETH2010/20	23.56	27.52	No Ct	25.26	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
GHA2010/01	20.71	19.69	No Ct	20.09	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
GHA2010/02	15.1	19.49	No Ct	21.48	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
GHA2010/03	30.8	32.07	No Ct	32.09	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
GHA2010/04	12.55	10.09	No Ct	12.49	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
GHA2010/05	14.31	15.89	No Ct	15.57	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
SEN2007/01	11.74	14.26	No Ct	15.47	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
SEN2007/02	12.92	16.86	No Ct	15.72	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
SEN2007/03	28.19	31.46	No Ct	28.46	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
SEN2007/04	27.2	29.28	No Ct	21.54	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
SEN2007/05	20.59	23.06	No Ct	17.59	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
ETH2010/03	16.82	14.24	No Ct	No Ct	No Ct	12.57	No Ct	No Ct	No Ct	No Ct	No Ct
ETH2010/04	13.43	9.88	No Ct	No Ct	No Ct	7.78	No Ct	No Ct	No Ct	No Ct	No Ct
ETH2010/05	17.8	16.91	No Ct	No Ct	No Ct	12.2	No Ct	No Ct	No Ct	No Ct	No Ct
ETH2010/06	19.4	15.8	No Ct	No Ct	No Ct	14.67	No Ct	No Ct	No Ct	No Ct	No Ct
ETH2010/07	19.44	20.62	No Ct	No Ct	No Ct	25.07	No Ct	No Ct	No Ct	No Ct	No Ct
KEN2007/01	20.17	20.91	No Ct	No Ct	No Ct	22.3	No Ct	No Ct	No Ct	No Ct	No Ct
SPA1987/01	12.36	15.93	No Ct	No Ct	No Ct	18.46	No Ct	No Ct	No Ct	No Ct	No Ct
ETH2010/19	15.97	22.39	No Ct	No Ct	No Ct	No Ct	No Ct	23.9	No Ct	No Ct	No Ct
SEN2007/06	14.77	19.09	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	24.56	No Ct	No Ct
ETH2010/10	23.61	21.59	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	22.82	No Ct
ETH2010/11	18.31	18.84	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	19.04	No Ct
ETH2010/14	21.05	24.69	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	25.41
ETH2010/15	21.95	22.46	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	28.79
ETH2010/16	20.47	21.53	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	21.17
GAM2009/0	14.48	17.39	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	20.15
GAM2009/0	14.16	15.89	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	18.59
KEN2006/01	15.89	25.86	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	20.27
Vaccine											
ETH2010/02	11.62	10.8	No Ct	27.42	17.27	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
ETH2010/09	21.11	20.32	No Ct	No Ct	No Ct	No Ct	24.39	No Ct	29.2	No Ct	No Ct
ETH2010/18	6.9	9.33	No Ct	No Ct	No Ct	No Ct	11.24	No Ct	No Ct	No Ct	23.04
SENvvv1/09	18.87	17.98	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	19.16

Seg-2 of isolates indicated with asterisk (*) was sequenced to confirm the typing results.

Table 4.5: Specificity of AHSV group specific assays (Seg-1 and Seg-3).

Virus species-serotype	Origin	ORC reference number	Seg-1	Seg-3
EEV-1	RSA	RSA1967/03	No Ct	No Ct
EEV-2	RSA	RSA1971/06	No Ct	No Ct
EEV-3	RSA	RSA1974/06	No Ct	No Ct
EEV-4	RSA	RSA1976/03	No Ct	No Ct
EEV-5	RSA	RSA1976/06	No Ct	No Ct
EEV-6	RSA	RSA1991/03	No Ct	No Ct
PHSV	Peru	PER1997/01	No Ct	No Ct
EHDV-1	USA	USA2001/03	No Ct	No Ct
EHDV-5	Australia	AUS1977/01	No Ct	No Ct
EHDV-7	Australia	AUS1981/06	No Ct	No Ct
EHDV-7	Israel	ISR2006/01	No Ct	No Ct
BTV-16	RSA	RSArrrr/16	No Ct	No Ct
BTV-2	India	IND1982/01	No Ct	No Ct
BTV-9	Turkey	TUR1998/04	No Ct	No Ct
BTV-8	UK	UKG2007/64	No Ct	No Ct
BTV-1	RSA	RSArrrr/01	No Ct	No Ct
BTV-12	RSA	RSArrrr/12	No Ct	No Ct
C. sonorensis			No Ct	No Ct
Uninfected BHK			No Ct	No Ct
Uninfected Vero			No Ct	No Ct
Uninfected KC cells			No Ct	No Ct
Uninfected horse blood			No Ct	No Ct

Representatives of different serotypes and topotypes from four different *Orbivirus* species (EEV, PHSV, EHDV and BTV) were tested to confirm the specificity of assays to recognise AHSV dsRNA. Further details on these isolates can be accessed from ORC http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm (Mertens & Attoui, 2011). EEV = Equine encephalosis virus; PHSV = Peruvian horse sickness virus; EHDV = Epizootic haemorrhagic disease virus; BTV = Bluetongue virus.

Three out of six field isolates of AHSV-9 produced positive signal at similar Ct values in both of the serogroup specific assays while the remaining three were detected earlier by Seg-1 based test (Table 4.4). This observation is contrary to the results with the reference strain of AHSV-9 (PAKrah9/03), which was detected earlier by the Seg-3 test. This discrepancy may indicate minor nucleotide variation within Seg-1 and Seg-3 between different isolates of the virus tested.

When dsRNA of other closely related *Orbivirus* species (EEV, PHSV, EHDV and BTV) were tested for amplification with the AHSV group-specific assays, a “no Ct” result was

consistently generated (Table 4.5). Similarly, no amplification was recorded in samples containing nucleic acid of *C. sonorensis,* uninfected horse blood, or cell culture supernatants (KC cells, BHK and Vero cells).

In silico analysis of sequence data of Seg-1 and 3 of other orbiviruses (EEV, Peruvian horse sickness virus (PHSV), Eubenangee Viruses (EUBV), Umatilla virus (UMAV), Pata virus (PATAV) EHDV and BTV) indicated that neither primers nor probes of the group specific AHSV assays described would bind to or act as primers to amplify their RNA.

4.2.4.2: Seg-2 based (type-specific) assays

Initially, the specificity of all 'type' specific (Seg-2 based) RT-PCR assays was tested with the nine reference strains of AHSV, characterised by full genome sequence analysis (Chapter 3).

All of the reference strains were successfully detected in the homologous assays, indicating their ability to detect the targeted template. However, when the heterologous assays were tested, some non-specific amplification was recorded (Table 4.6). Four reference strains (RSArrah/01, RSArrah/04, RSArrah/05, RSArrah/08) were detected (cross-reactions) by one or more of the assays designed to detect other AHSV types. To confirm these results another, separately maintained set of the historical reference strains were also tested, with the same results. However, *in sili*co analysis of the primer and probe binding sites on Seg-2 sequences from the reference strains did not provide any explanation for these apparent cross-reactions. The possibility of non-specific amplification of non-targeted AHSV serotypes was therefore excluded.

It was considered likely that the original reference strains of AHSV that cross-reacted contained mixtures of more than one AHSV serotype. To test this hypothesis and to ensure the specificity of the assays for typing AHSV in diagnostic samples, a panel of nine plaque purified AHSV strains (O'Hara *et al.*, 1998) (Chapter 5), were also tested with the Seg-2 based typing assays. In each case amplification was only observed with the homologous serotype (Table 4.4). The lack of heterologous amplification indicates that there is no 'cross-detection' of Seg-2 sequences of the AHSV types in these assays, and it was therefore concluded that these assays are 'type-specific'. It was concluded that the earlier cross-reactions were due to cross-contamination of the original reference strains, due to very high sensitivity of the methodology. These cross-contaminants had previously gone largely unnoticed.

Evaluation of the diagnostic specificity of the nine typing assays using field strains of all nine AHSV serotypes produced positive signals only in the homologous assays (Table 4.4). These RT-PCR results were confirmed by sequencing for majority of the isolates tested (Table 4.4).

The AHSV-9 vaccine strain produced by the National Veterinary Research Institute, in Senegal (SENvvv1/09), and the three vaccines (AHSV-2, AHSV-5 and AHSV-9) manufactured at the National Veterinary Institute, Ethiopia (ETH2010/02, ETH2010/09 and ETH2010/18, respectively), were also tested with the panel of novel real-time RT-PCR typing assays. Although, positive results were obtained in each case with the homologous assays, signals indicating heterologous serotypes were also generated for each of the Ethiopian vaccine strains (Table 4.4). The AHSV-2 vaccine strain (ETH2010/02) was also found to AHSV-3; the AHSV-7 vaccine strain (ETH2010/09) also contained AHSV-5, and AHSV-5 was also found in the AHSV-9 vaccine strain (ETH2010/18).

Table 4.6: Typing assays specificity results with AHSV historical reference strains.

	SRT-1	SRT-2	SRT-3	SRT-4	SRT-5	SRT-6	SRT-7	SRT-8	SRT-9
RSArrah/01	20/19			28/No Ct					
RSArrah/02		20/20							
RSArrah/03			19/19						
RSArrah/04	30/No Ct			19/42					
RSArrah/05			28/29	34/31	31/32				
RSArrah/06						19/20			
KENrrah/07							24/25		
RSArrah/08					27/32			20/21	
PAKrrah/09									19/23

Each of the original AHSV reference strains was tested with homologous and heterologous typing assays. Each of the strains was successfully detected by its homologous assays (highlighted in green) but non-specific amplification was recorded in some of the strains when tested with heterologous assays (highlighted in blue).

These real-time RT-PCR data were confirmed by Seg-2 sequencing of the mixed Ethiopian vaccine strains and two distinctive consensus sequences were generated in each case. Comparison of these data to the Seg-2 dataset from the nine AHSV reference strains (Chapter 3) confirmed the real-time RT-PCR typing results. Seg-2 from ETH2010/02 (the 'AHSV-2' vaccine) grouped with RSArrah/02 (AHSV-2) and RSArrah/03 (AHSV-3) showing 96 and 99.7% nucleotide identities (suggesting that the contamination had been caused by the reference strain or a very similar virus). Seg-2 of ETH2010/09 ('AHSV-7' vaccine) grouped with KENrrah/07 (AHSV-7) and RSArrah/05 (AHSV-5) with 99% and 98.1% nucleotide identities respectively, indicating contamination with an AHSV-5 that related to but distinct from the reference strain. Seg-2 of ETH2010/18 (AHSV-9 vaccine) grouped with PAKrrah/09 (AHSV-9) and RSArrah/05 (AHSV-5) with 96.6% and 99.4% nucleotide identities, indicating that the contaminant was again closely related to the AHSV-5 reference strain. Although these close relationships to the reference strains are considered most likely to reflect laboratory derived contamination, it is notable that most of the reference strains were isolated in South Africa, and they could potentially reflect

mixed strains that originally co-circulated in the field. Further phylogenetic analyses might resolve these two posibilities.

Nucleic acid of representative virus host species (uninfected horse blood and *C. sonorensis*) and cell culture supernatants (KC cells, BHK and Vero) gave 'no Ct' values in any of the nine type specific assays.

4.2.5: Analytical sensitivity and efficiency

Analytical sensitivity refers to the detection limit of small amounts of the target nucleic acid. It can be measured by assessing the limit of detection of RNA and infectivity.

Efficiency (E) of a PCR reaction can be explained as a rate at which a PCR amplicon is generated. A PCR reaction (assay) is 100% efficient when its amplicon doubles in quantity during a single round of the geometric phase of the amplification. Efficiency of a real-time PCR assay can be calculated in following equation: $E = (10^{-1/slope}-1) \times 100$

The slope is calculated from linear regression of the Ct values versus log of input of nucleic acid. A standard curve slope of -3.32 indicates a PCR assay with 100% efficiency.

4.2.5.1: Seg-1 and Seg-3 based (group-specific) assays

The analytical sensitivity of the serogroup specific assays (Seg-1 and Seg-3) was assessed separately using two independent methods, firstly with a dilution series of AHSV viruses (all nine serotypes) and secondly with a dilution series of the quantified dsRNA genome of AHSV-1.

A tenfold dilution series of the nine monotypic (plaque purified) AHSV reference strains were prepared in cell culture medium, startying at a titre of 10^5 $TCID_{50}$/ml, down to 1

$TCID_{50}$/ml (titrated in BHK cells). The viral dsRNA was extracted from the dilution series using BioRobot Universal (Qiagen), as described in section 2.4.2 and tested in duplicate with each of the optimised assays. Seg-1 and Seg-3 of all AHSV serotypes were detected in the respective assays, down to the lowest dilution of 1 $TCID_{50}$/ml (Table 4.7). Two hundred and forty microliters of the final dilution (1 $TCID_{50}$/ml) had been used for the RNA extractions, and the resulting RNAs were resuspended in 50µl. A total of 6 µl of each RNA preparation was then used in each replicate assay. Consequently each 'lowest-dilution' RT-PCR assay contained RNA equivalent to 0.029 of a $TCID_{50}$, which was consistently detected as positive (with a Ct value of 33.77 in the Seg-1 specific assay, and 35.46 in the Seg-3 specific assay, using AHSV-1 (RSArah1/03) (Table 4.7)).

The dilution curves for each of the nine serotypes in their respective assays were drawn by plotting mean Ct values against the corresponding dilution of the original virus dilution-series. These were used to estimate efficiency from the curves' slopes (as described in section above) and to perform linear regressions analyses of the dilutions to obtain the coefficient of determination (R^2).

Efficiency rates for both of the group-specific assays (Seg-1 and Seg-3) varied depending on the AHSV serotype and in the Seg-1 based assay was between 98 and 108%, while the range in the Seg-3 based assay was wider, between 101 and 115% (Table 4.7). The 'slope' in both of the group specific assays (which is a measure of an assay's efficiency) showed only minor variations, from -3.0 to -3.3, with a good linear correlation ($R^2 > 0.98$) for all AHSV serotypes (Table 4.7; Figure 4.1). The analysis of variance (ANOVA) showed no significant difference ($p < 0.05$) between the individual slopes in either of the group specific assays (Figure 4.2).

The analytical sensitivity of both group-specific assays (Seg-1 and Seg-3) was also determined by detection of known amounts of AHSV-1 (RSArah1/03) dsRNA (dsRNA standards). Preparation of these standards is described in Chapter 2, section 2.8.1. For this purpose a 10-fold dilution series of dsRNA (10^9 to 10^0 copies per µl) was made in a sample of RNA extracted from uninfected BHK supernatant, then tested in quadruplicate. Six microliters of each dilution were used in each RT-PCR reaction.

The group specific real-time RT-PCR assays detecting Seg-1 and Seg-3 could amplify (identify as positive) all four repeats containing 60 RNA copies per reaction. At the next lower dilution of the virus (6 copies per reaction) the viral RNA was only detected in two out of the four in the Seg-1 assay, and one out of four in the Seg-3 assay. Reactions containing 60 copies of purified AHSV-1 genomic RNA gave a Ct value of 33.96 in the Seg-1 specific assay (and 35.16 in the Seg-3 specific assay) (Table 4.8) approximately equivalent to the assays containing RNA extracted from 0.029 $TCID_{50}$ of the virus dilution series (see above). This suggests that in both assays 60 copies of RNA are roughly equivalent to 0.029 $TCID_{50}$ of the virus (or 1 $TCID_{50}$ is equivalent to 2068 copies of the virus genome or virus particles). Therefore detection of the RNA (down to between 6 to 60 copies) is much more efficient (at least 2000 fold) than detection of viral infectivity, which cannot go below 1 $TCID_{50}$.

In previous studies of BTV infectivity in BHK cells it was found that ~1000 copies of the disaggregated virus particle were equivalent to 1 $TCID_{50}$ (Mertens *et al.*, 1987). The slightly lower infectivity to particle ratio calculated here may reflect RNA losses during its extraction from the virus particle preparation.

The efficiency rates for the Seg-1 assay, of 101% and for the Seg-3 assay of 96% were estimated over eight (10^1-10^8) and seven (10^1-10^7) log dilutions for the respective

systems. Over these dilution ranges a linear dynamic with R^2 exceeding 0.99 was observed for both of the assays (Table 4.8 and Figure 4.3).

Table 4.7: Analytical sensitivity and efficiency of group specific (Seg-1 and Seg-3) and type specific (Seg-2) assays with serially diluted virus.

Assay	Isolate	Slope	Efficiency (%)	R^2	$TCID_{50}$/ml					
					10^5	10^4	10^3	10^2	10^1	10^0
Seg-1	RSArah1/03	-3.296	101.1	0.998	17.36	21.06	24.17	27.38	31.09	33.77
Seg-1	RSArah2/03	-3.346	99.0	0.997	16.56	20.65	23.17	26.89	29.83	33.73
Seg-1	RSArah3/03	-3.361	98.4	0.994	21.1	25.53	28.3	32.41	35	38.12
Seg-1	RSArah4/03	-3.303	100.8	0.993	16.07	19.91	23.71	27.2	29.51	32.73
Seg-1	RSArah5/03	-3.138	108.3	0.991	19.02	23.09	26.69	29.66	32.06	35.01
Seg-1	RSArah6/03	-3.243	103.4	0.999	20	22.9	26.37	29.62	32.59	36.24
Seg-1	KENrah7/03	-3.218	104.5	0.992	17.22	20.41	24.91	27.66	30.3	33.26
Seg-1	RSArah8/03	-3.228	104.1	0.999	17.08	20.37	23.53	27.08	30.13	33.11
Seg-1	PAKrah9/03	-3.177	106.4	0.985	16.73	21.09	24.77	28.04	30.08	32.92
Seg-3	RSArah1/03	-3.265	102.4	0.999	19.13	22.63	25.85	28.9	32.49	35.46
Seg-3	RSArah2/03	-3.192	105.7	0.995	16.02	20.23	23.56	26.29	29.06	32.52
Seg-3	RSArah3/03	-3.284	101.6	0.995	22.02	25.71	28.54	31.91	34.55	39.03
Seg-3	RSArah4/03	-3.304	100.7	0.993	16.09	19.93	23.73	27.23	29.53	32.76
Seg-3	RSArah5/03	-3.055	112.5	0.998	20.52	23.18	26.74	29.87	32.86	35.47
Seg-3	RSArah6/03	-3.282	101.7	0.979	19.81	23.3	26.12	30.01	34.63	35.21
Seg-3	KENrah7/03	-3.244	103.4	0.999	16.17	19.77	22.89	26.29	29.13	37.9*
Seg-3	RSArah8/03	-3.013	114.8	0.994	16.41	19.41	22.79	26.4	28.22	31.49
Seg-3	PAKrah9/03	-3.297	101.1	0.985	12.63	14.26	17.88	21.62	24.41	28.87
Seg-2	RSArah1/03	-3.587	90.0	0.999	18.3	21.83	25.82	29.06	32.58	36.31
Seg-2	RSArah2/03	-3.598	89.7	0.994	15.27	18.38	23.24	26.72	29.71	32.96
Seg-2	RSArah3/03	-3.432	95.6	0.982	23.44	26.95	31.99	33.99	37.08	34.97*
Seg-2	RSArah4/03	-3.529	92.0	0.967	17.1	19.95	26.61	29.02	30.65	34.9
Seg-2	RSArah5/03	-3.111	109.6	0.962	22.48	27.43	31.46	34.53	36.03	38.48
Seg-2	RSArah6/03	-3.595	89.7	0.961	21.34	24.31	30.73	32.58	35.18	No Ct
Seg-2	KENrah7/03	-3.196	105.5	0.995	19.24	22.69	26.78	29.34	32.24	35.37
Seg-2	RSArah8/03	-3.041	113.2	0.993	18.41	21.05	25.12	28.13	30.86	33.21
Seg-2	PAKrah9/03	-3.252	103.0	0.982	11.73	16.9	20.5	22.73	25.83	28.69

Both group specific assays (Seg-1 and Seg-2) detected all nine serotypes of AHSV diluted to down to 1 $TCID_{50}$/ml (last dilution analysed). Similarly most of the type specific assays also detected the homologous AHSV type diluted to down to 1 $TCID_{50}$/ml (last dilution analysed (apart from the AHSV-6 assay which could detect the virus down to 10 $TCID_{50}$/ml). The slope of the linear regression, its correlation (R^2) and efficiency were also calculated for each virus serotype in each assay. Values marked with asterisk (*) were not used for the calculations.

Table 4.8: Analytical sensitivity and efficiency of group specific (Seg-1 and Seg-3) assays with serially diluted dsRNA standards (RSArrah/01).

Isolate	Slope	Eff (%)	R^2	Copy number								
				$6x10^8$	$6x10^7$	$6x10^6$	$6x10^5$	$6x10^4$	$6x10^3$	$6x10^2$	$6x10^1$	6
RSArah1/03	-3.3	100.9	0.998	10.37	14.06	17.39	20.54	23.83	27.23	30.31	33.96	37.89*
RSArah1/03	-3.415	96.3	0.998	10.14*	14.49	17.98	21.27	24.58	28.01	31.4	35.14	39.01*

Mean values of four repeats per each dilution are presented. dsRNA diluted down to 60 copies (equivalent to 60 virus particles) per reaction could be detected with 100% analytical sensitivity (in all four repeats), while a six copies of the virus per reaction were detected in 50% of the repeats (2 out of 4) in the Seg-1 assay and 25% (1 out of 4) in the Seg-3 assay. The slopes for each assay have been estimated and assay's efficiency and correlation (R^2) calculated. Values marked with asterisk (*) were not used for the calculations. Eff = Efficiency.

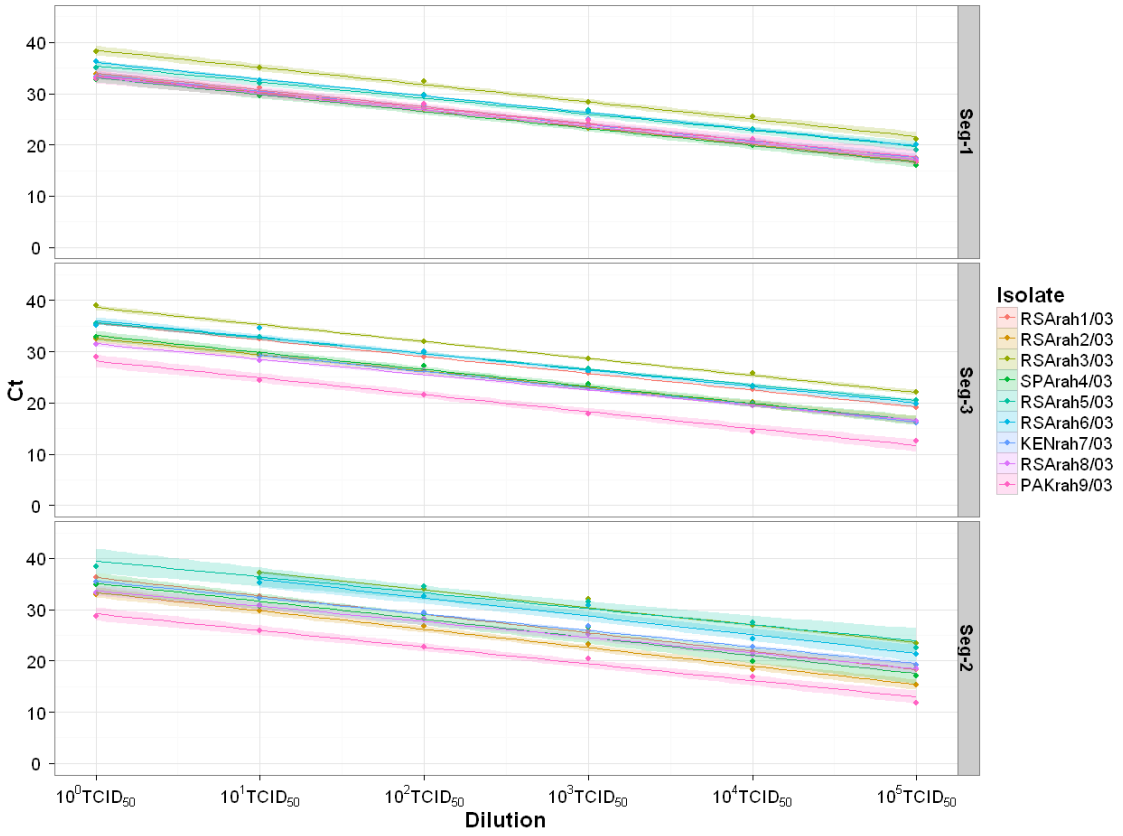


Figure 4.1: Standard curves for CT value against virus titre for AHSV group and type specific RT-PCR assays.
Comparison of standard curves generated by plotting Ct values against RNA extracted from log virus dilution with corresponding 95% confidence intervals (CI). All nine monotypic reference strains were analysed for both group specific assays and the homologues strain for each of the type specific assays.

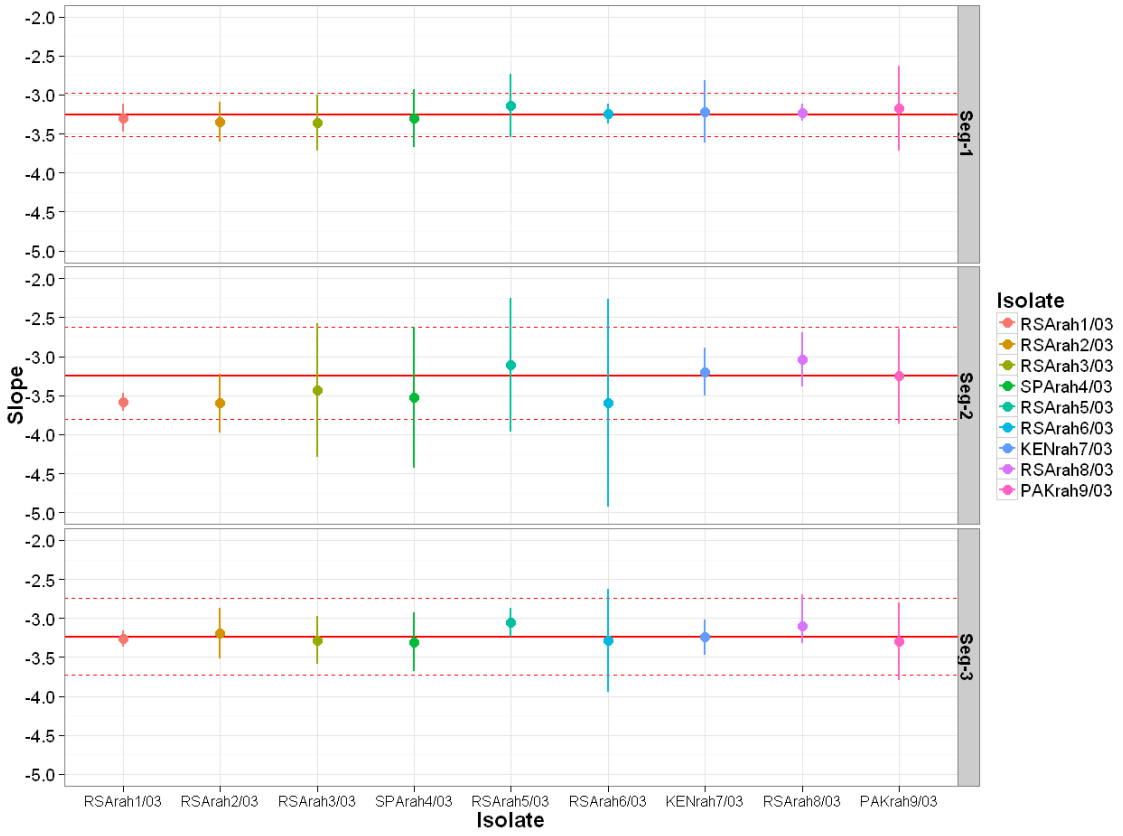


Figure 4.2: Slopes values in in relation to the slope of the common regression line for group and type specific RT-PCR assays.
Comparison of slopes of the nine reference strains to the respective common regression line in group specific (Seg-1 and Seg-3) and type specific (Seg-2) assays with corresponding 95% CI. Slopes of all nine serotypes of AHSV are highly similar in each of the group specific or individual type specific assays respectively. Solid red line represents common regression line while dotted lines mark defines the 95% CI.

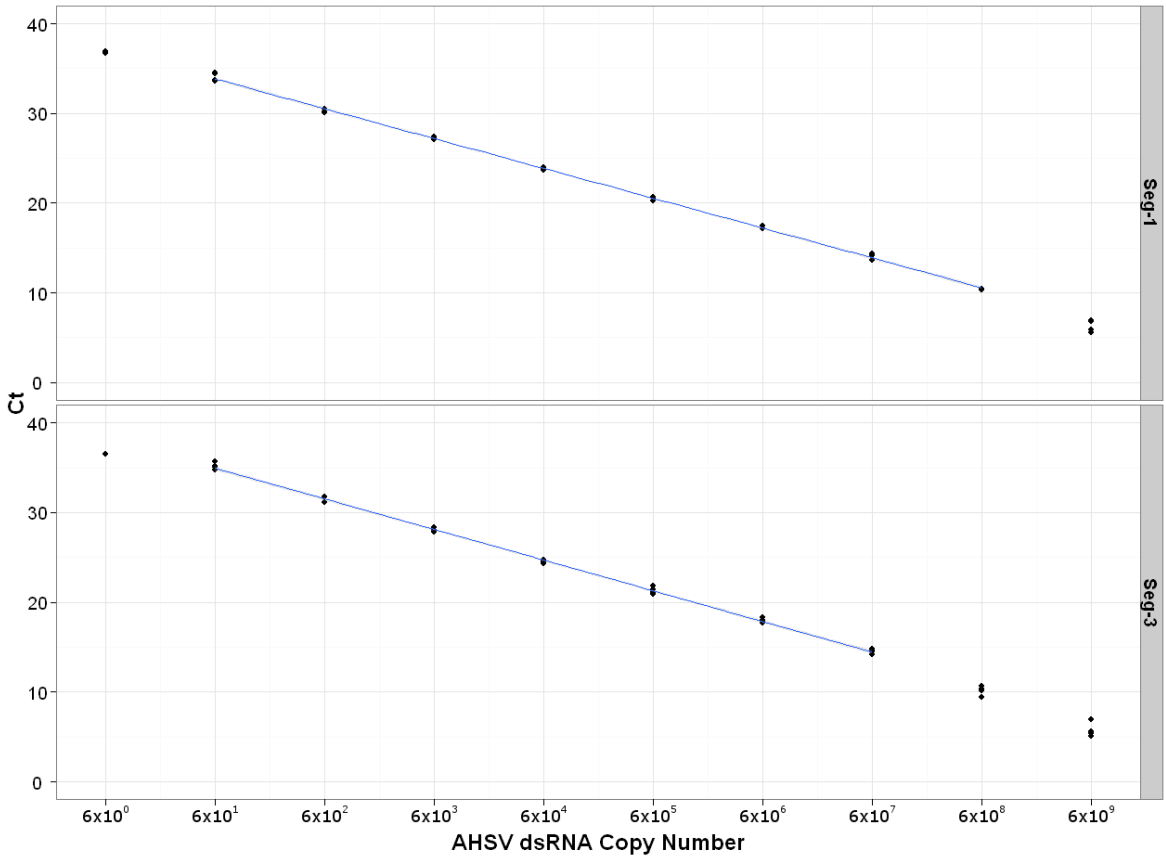


Figure 4.3: Standard curves for CT value against RNA copy number for Seg-1 and Seg-3 RT-PCR.
Standard curves generated by plotting Ct values against corresponding log copy number of viral dsRNA (RSArrah/01) in Seg-1 and Seg-3 assay. Each dilution was tested in quadruplicates. Non-linearity of lowest and highest dilutions were excluded from the regression analysis.

4.2.5.2: Seg-2 based assays (serotype assays)

The analytical sensitivity of each typing assays (Seg-2 based) was also tested using a duplicate 10-fold dilution series of the homologous AHSV reference strain, starting at a titre of 10^5 $TCID_{50}$/ml (titrated in BHK21 cells). In eight of the typing assays a Ct value was obtained with all five dilutions of the homologous viruses, down to 1 $TCID_{50}$/ml of virus (0.017 $TCID_{50}$/ assay), although the AHSV-6 specific assay gave positive Ct values down to 10 $TCID_{50}$/ml (0.17 $TCID_{50}$/ assay), (Table 4.7).

Efficiency rates calculated from the 10 fold dilutions series of virus, varied between 90-110% depending on the assay (Table 4.7), which reflects the range of slope values (between -3 and -3.6). In all 9 Seg-2 detecting assays, strong linearity was found (R^2 > 0.98) (Table 4.7 and Figure 4.1). No statistical difference ($p<0.05$) were identified between slopes using ANOVA analysis (Figure 4.2).

4.3: Discussion

Sequence analysis of the full-genomes of the AHSV reference strains (Chapter 3) and Seg-2 from multiple field strains (Chapter 6), provided a basis for development of real-time RT-PCR assays to detect and type AHSV isolates. This is the first development of TaqMan technology based, real-time RT-PCR systems for AHSV typing. The typing assays (Seg-2 based) were validated alongside group-specific assays detecting highly conserved regions of Seg-1 and Seg-3. The combination of group and type specific diagnostic systems provides a complete set of tools for the rapid and accurate detection of AHSV and identification of its type.

All of the assays were able to detect a wide range of reference, field and vaccine strains confirming their diagnostic sensitivity. Both group specific assays (Seg-1 and Seg-3) are shown to be specific to AHSV. There was also no evidence of cross-amplification of other serotypes of the virus in any of the type-specific assays (Seg-2). Therefore this set of real-time RT-PCR assays for detection and typing of AHSV can be used for surveillance and molecular epidemiology studies of the AHSV, as well as providing a set of tools for monitoring / surveillance of AHSV epidemics.

The ability to detect and type AHSV with precision is necessary for any attempt to understand the epidemiology of the virus. Accurate, high throughput AHSV detection and

typing tests can aid tracing of the virus movements and therefore to increase our ability to predict future outbreaks of the disease.

All of the field strains investigated in this study were successfully detected with both of the group specific assays. However, some isolates of AHSV were detected with different sensitivities (by the assays targeting Seg-1 and Seg-3). This may indicate changes in oligonucleotide binding regions, between different isolates, even though both of the group specific assays were designed to target highly conserved regions of the most invariable genome segments, encoding structural proteins VP1 and VP3 of AHSV (Chapter 3). It is possible that strains of AHSV will arise in the field that have further changes that significantly reduce the efficiency of one or other of these assays. This would require a redesign of the assay that had failed, in order to maintain its effectiveness. However, it is considered unlikely that both group specific assays would fail simultaneously and their use in tandem would still maintain diagnostic capability.

AHSV infection generates a relatively low level of viraemia in horses (up to ~10^5 $TCID_{50}$/ml) and an even lower circulating titre in donkeys and zebras (< 10^3 $TCID_{50}$/ml) (Hamblin *et al.*, 1998). Studies of analytical sensitivity reported here for the RT-PCR assays, indicate that a viraemic animal would be detected reliably using either the group-specific or type-specific assays.

All of the assays developed here (both group and type-specific) have been shown to be highly efficient, doubling amplicon quantity during each round of amplification in the geometric phase of the reaction. This was true for all nine serotypes of AHSV in both the group-specific assays and the respective type-specific assays (Figure 4.1 and 4.3). The group-specific assays (Seg-1 and Seg-3) can be used to quantify the amounts of viral dsRNA, even at very low concentrations (down to 60 copies of viral dsRNA per reaction) (Figure 4.3).

The type-specific real-time RT-PCR assays are the most sensitive and reliable method for typing AHSV, that is currently available (Koekemoer, 2008; Sailleau *et al.*, 2000), while the group-specific assay can detect similar levels of RNA to other published real-time AHSV detection methods (Fernandez-Pinero *et al.*, 2009; Monaco *et al.*, 2011; Quan *et al.*, 2010; Rodriguez-Sanchez *et al.*, 2008).

The group-specific assays target highly conserved genome segments (Seg-1 and Seg-3) which have some of the lowest nucleotide substitution rates per site of all AHSV genome segments (Table 3.22). Also, the gene coding nucleotide sequences of Seg-1 and Seg-3 are under strong purifying selection (Chapter 3, section 3.3.5) indicating an evolutionary pressure, to maintain the biological functions and sequences of these genes. These properties of Seg-1 and Seg-3 make them suitable as targets for a robust AHSV group-specific diagnostic system. However, the relatively small number of AHSV isolates that were available for this study (n=42), especially for serotypes 1, 3 and 5, has implications for the wider validation of these assays, particularly for the type-specific (Seg-2) based assays.

The diagnostic specificity of the nine typing assays described here, relates primarily to their inability to detect Seg-2 of non-homologous types, while still detecting all of the relatively small number of available isolates from the homologous AHSV type. If a novel isolate of the virus is identified (e,g, by the group-specific assays) that fails to amplify using the typing assays, it will be sequenced (Seg-2) to provide a basis for further development of these tests. Similarly problems were addressed with relevant conventional RT-PCR assays detecting Seg-2 of BTV (Maan *et al.*, 2012).

Each of the assays described is already being used for diagnostic or research purposes in a wide range of host and insect vector species, by the reference and research groups at the Pirbright Institute. The Seg-1 based group-specific assay has been used for detection of

AHSV-4 dsRNA in blood from IFNAR-/- mice (Castillo-Olivares *et al.*, 2011) while replication of AHSV-4 in oral fed *C. sonorensis* has been assessed using the Seg-3 specific assay (Veronesi, 2012). The AHSV serotypes which have caused recent multi-serotype outbreak in Ethiopia (2010) (Aklilu *et al.*, 2012) were characterised using the Seg-2 based typing assays and results were confirmed by sequencing (Chapter 6). The protocol for all of the assays has been optimised to maximise sensitivity and it is easy to follow. The same thermal profile is used for each of the 11 group and type assays, so specific investigations can be undertaken on a single machine at the same time.

In conclusion, these studies have provided an easy to use 'TaqMan' real-time RT-PCR based methods, which represent fast, robust and reliable tools for the detection and identification (serotyping) of AHSV. They provide a basis for the design and timely implementation of control measures for AHSV, including vaccination programmes. These diagnostic methods (particularly if linked to sequencing and phylogenetic studies) will also improve our understanding of the prevalence, distribution and movements of AHSV during epidemiology studies of the virus.

Chapter 5: Propagation and characterisation of monotypic reference strains of AHSV

5.1: Introduction

A set of nine strains of AHSV, each representing a different serotype of the virus, were originally isolated in South Africa at Onderstepoort Veterinary Institute (OVI) from clinical samples collected in South Africa, Kenya and Pakistan (O'Hara *et al.*, 1998). They were then made available, as AHSV 'reference strains', to scientists investigating the virus in different research centres working on this pathogen (Aguero *et al.*, 2008; Hamblin *et al.*, 1992; Hamblin *et al.*, 1990b; House *et al.*, 1990; O'Hara, 1994; Rodriguez-Sanchez *et al.*, 2008; Sailleau *et al.*, 2000). This set of samples was subsequently used for wide range of research activities including investigations of pathogenicity of the virus (Laegreid *et al.*, 1993; O'Hara, 1994), development of diagnostic serum (Hamblin *et al.*, 1992; Hamblin *et al.*, 1990b; House *et al.*, 1990) and detection assays (Aguero *et al.*, 2008; Moulay *et al.*, 1995; Sailleau *et al.*, 2000).

However during the validation of type-specific real-time RT-PCR assays for AHSV, it was discovered that some of the original reference virus strains (from the ORC at the Pirbright Institute) contained Seg-2 from more than one AHSV serotype (Chapter 4, section 4.2.4.2) and are therefore mixed virus preparations. These included the reference strains of AHSV-1, AHSV-4, AHSV-5 and AHSV-8. Previous studies have also reported detection of mixed strains in AHSV-1 and AHSV-2 (Potgieter *et al.*, 2009; Sailleau *et al.*, 2000). It was therefore decided that new 'plaque cloned' and monotypic reference strains were required, for all nine AHSV serotypes.

This chapter describes selection and generation of a new, monotypic, set of nine AHSV reference strains and their characterisation by sequencing of genome segment 2 and 6, encoding outer capsid proteins VP2 and VP5.

5.2: Results

5.2.1: The origin and nomenclature of the viruses

Three times plaque purified AHSV viruses (clones) for each AHSV serotype, were selected from stocks created by Dr Rachel O'Hara during her PhD project (O'Hara, 1994). These clones were deposited at the Pirbright Institute. Most of the plaque purified strains originated from the AHSV reference strains isolated in South Africa (AHSV-1 to 3 and AHSV-5 to 9) while AHSV-4 originated from the 1987 epidemics in Spain (AHSV-4) (Chapter 1, section 1.7.1) (O'Hara, 1994). The nomenclature for the viruses which were selected as the new monotypic reference strains is listed in Table 5.1.

Table 5.1: Nomenclature of the nine monotypic plaque cloned reference strains.

AHSV type	Virus original designation	Designation of individual viral clones as assigned by Dr R. O'Hara	ORC designation
AHSV-1	29/62	AHSV-1 (C) MB3/BHK8 27/01/91	RSArah1/03
AHSV-2	82/61	AHSV-2 (F) MB3/BHK5 11/12/91	RSArah2/03
AHSV-3	13/63	3 113 08/02/93	RSArah3/03
AHSV-4	Ricarda	clone 213 18/01/93	SPArah4/03
AHSV-5	30/62	AHSV-5 (F) MB3/BHK5 27/01/92	RSArah5/03
AHSV-6	39/63	AHSV-6 (C) MB3/BHK4 270192	RSArah6/03
AHSV-7	31/62	AHSV-7 (F) MB4/BHK7 300692	KENrah7/03
AHSV-8	10/62	AHSV-8 (G) MB3/BHK7 300692	RSArah8/03
AHSV-9	90/61	9 113 04/08/92	PAKrah9/03

5.2.2: Characterisation of monotypic AHSV reference strains using Seg-2 specific real-time RT-PCR

The monotypic character of each of the chosen viral clones was tested before their propagation and storage, using the real-time RT-PCR assays developed as described in Chapter 4. Most of the virus clones were found to be monotypic. However, one of the clones originating from AHSV-8 (10/62) gave a positive RT-PCR signal with the assay targeting Seg-2 of AHSV-5 (data not shown). Another clone of AHSV-8 was therefore chosen and tested by real-time RT-PCR to ensure that each serotype of the virus is represented in the monotypic set AHSV reference strains.

Following selection, the viruses were passaged two times in BHK cells (as described in Chapter 2, section 2.3). After propagations, the supernatant from infected cells for each of the respective clones was tested with the set of the nine serotyping assays (Chapter 4). Positive amplification for each individual virus was recorded with type-specific assay only and in each case, with an assay detecting homologous type of the virus. No amplification with heterologous assays was detected in any of the viruses tested, confirming that the selected and propagated clones of the virus are monotypic. The results of the real-time RT-PCR test after two passages of the virus in BHK cells are presented in Table 5.2.

5.2.3: Genetic characterisation of the monotypic AHSV reference strains

Seg-2 and Seg-6, encoding for the outer capsid proteins, were sequenced for each of the new plaque cloned and monotypic reference strains. The sequence data were compared to the Seg-2 and Seg-6 sequence database, for all nine serotypes of AHSV described in Chapter 3 sections 3.2.3.2 and 3.2.3.6.

Table 5.2: Confirmation of the monotypic nature of the new AHSV reference strains.

AHSV isolate	Seg-2 Assay Type								
	-1	**-2**	**-3**	**-4**	**-5**	**-6**	**-7**	**-8**	**-9**
RSArah1/03	15.07	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
RSArah2/03	No Ct	12.42	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
RSArah3/03	No Ct	No Ct	13.69	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
RSArah4/03	No Ct	No Ct	No Ct	13.85	No Ct	No Ct	No Ct	No Ct	No Ct
RSArah5/03	No Ct	No Ct	No Ct	No Ct	11.46	No Ct	No Ct	No Ct	No Ct
RSArah6/03	No Ct	No Ct	No Ct	No Ct	No Ct	12.11	No Ct	No Ct	No Ct
KENrah7/03	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	14.46	No Ct	No Ct
RSArah8/03	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	12.88	No Ct
PAKrah9/03	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	13.1

Sequence identity levels between Seg-2 and Seg-6 of the original and new sets of reference strain viruses showed 100% identity between AHSV-1, AHSV-2, and AHSV-6 in both genome segments. Seg-2 was also identical in AHSV-3 and AHSV-9, and Seg-6 was identical in AHSV-5 and AHSV-8. However, Seg-2 of the AHSV-4, 5 7 and 8 strains and Seg-6 of the AHSV-3, 4, 7and 9 showed identities of ≥99.9% indicating a very close match and confirming their serotype (Table 5.3).

In each case, a clear match between the 'historical' and the 'new' monotypic reference strains as confirmed by phylogenetic analyses using a Maximum Likelihood method (Felsenstein, 1981) (Figure 5.1 and 5.2).

Table 5.3: Percentage of identities in Seg-2 and Seg-6 between the 'historical' and 'new' AHSV reference strains.

	RSArrah/01	RSArrah/02	RSArrah/03	SPArrah/04	RSArrah/05	RSArrah/06	KENrrah/07	RSArrah/08	PAKrrah/09
RSArah1/03	**100**/100								
RSArah2/03		**100**/100							
RSArah3/03			**100**/99.9						
SPArah4/03				**99.9**/99.9					
RSArah5/03					**99.9**/100				
RSArah6/03						**100**/100			
KENrah7/03							**99.9**/99.9		
RSArah8/03								**99.9**/100	
PAKrah9/03									**100**/99.9

Numeric values refer to percentage of nucleotide identities between analysed strains. Seg-2 identities are tabulated in bold face.

5.2.4: The new monotypic set of AHSV reference strains

The new set of the nine plaque cloned and monotypic reference strains have been stored in the ORC at the Pirbright Institute, UK (as described in Chapter 2 section 2.3) under codes listed in Table 5.1. They are now available to researchers and diagnosticians and details can be accessed via following link to the "RNAs and Proteins of dsRNA Viruses" web page (Mertens & Attoui, 2011) http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/AHSV-isolates.htm.

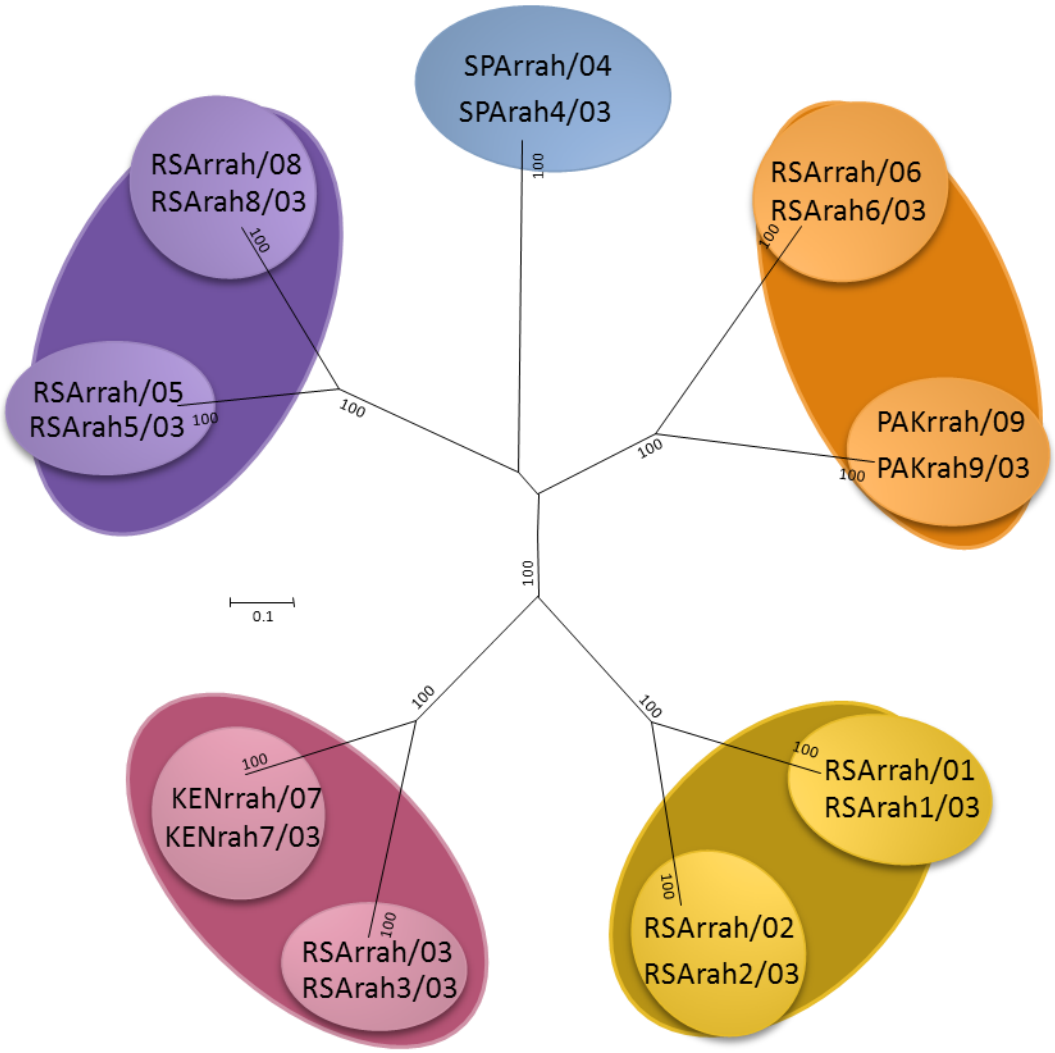


Figure 5.1: Phylogenetic analysis of Seg-2 nucleotide sequences of the 'historical' and 'new' AHSV reference strains.

The analysis was inferred using a Maximum Likelihood method, based on the General Time Reversible (GTR) model with a discrete Gamma distribution. The rate variation model allowed for some sites to be evolutionary invariable. The tree is drawn to scale, with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

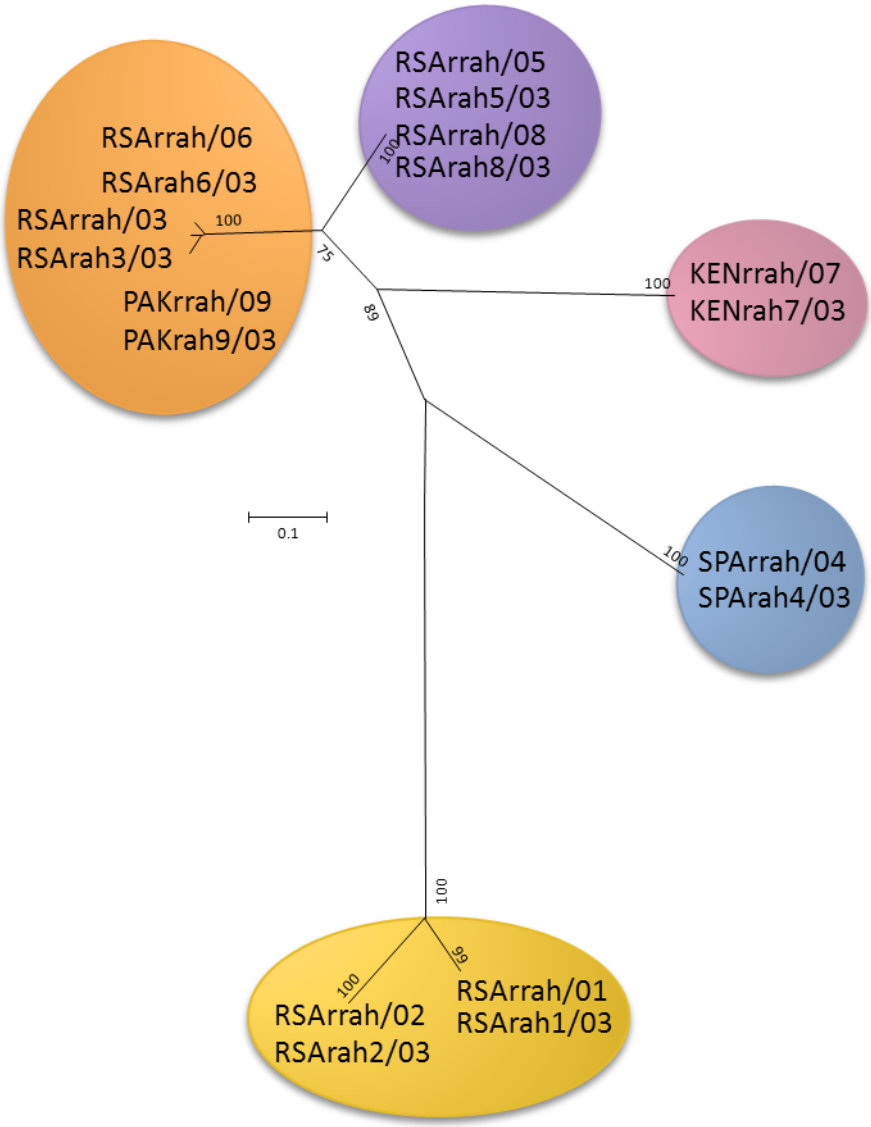


Figure 5.2 Phylogenetic analysis of Seg-6 nucleotide sequences of the 'historical' and 'new' AHSV reference strains.

The analysis uses a Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model (Jones *et al.*, 1992) with a discrete Gamma distribution. The tree is drawn with branch lengths measured in the numbers of substitutions per site with bootstrap of 1,000 replicates. The trees were generated in MEGA5 (Tamura *et al.*, 2011).

5.3: Discussion

A uniform set of African horse sickness viruses known as the 'the reference strains' were created and made available at the OIE Reference Centre for Bluetongue and African horse sickness OVI, South Africa (Potgieter *et al.*, 2003). Access to the viruses, representing each of the nine serotypes of AHSV, allowed scientists from different research and diagnostic institution to compare results of their investigations.

For this reasons, this set of AHSV strains has been wildly tested, including determination of the viruses' serotypes (House *et al.*, 1990; Koekemoer, 2008; Koekemoer *et al.*, 2000; Koekemoer & van Dijk, 2004; Sailleau *et al.*, 2000). However, no cross-reaction between different reference strains was reported, apart from a study which detected AHSV-4 in the AHSV-2 reference strain (Sailleau *et al.*, 2000). Also, a deep sequencing investigation of AHSV-1 reference strain reported the presence of four AHSV-3 genome segments (Seg-5, Seg-7, Seg-8 and Seg-10) in this virus isolate (Potgieter *et al.*, 2009).

In course of this study, real-time RT-PCR assays were developed optimised and validated to detect each type of AHSV (Chapter 5). During this process four of the different AHSV reference strains available at the Pirbright Institute (AHSV-1, AHSV-4, AHSV-5 and AHSV-8) were found to contain Seg-2 of heterologous AHSV serotypes, indicating a mix population of viruses (Chapter 5). AHSV-3 Seg-2 was not detected in the AHSV-1 reference strain, which is in agreement with the deep sequencing study, which found four other segments from the AHSV-3 reference strain (Potgieter *et al.*, 2009). However, we could not detect AHSV-4 Seg-2 in the AHSV-2 reference strain as previously reported (Sailleau *et al.*, 2000).

The AHSV historical reference strains have been used to raise type specific reference antiserum which were subsequently used as research and diagnostic reagents (Hamblin *et*

al., 1992; Hamblin *et al.*, 1990b; House *et al.*, 1990). The use of mixed strains for these purposes could potentially lead to inconclusive research and diagnostic results.

The use of mixed BTV challenge strains has previously been reported in animal experiments (Bréard *et al.*, 2011; Eschbaumer *et al.*, 2011). Had the contamination of the challenge strains remained undetected it could have resulted in misleading conclusions from animal experiments, and the apparent serological cross-reactions between serotypes. These incidents highlight the importance of quick, robust and convenient tests (like those described in Chapter 4) for detection and confirmation of the type of AHSV used for research, diagnosis and vaccination purposes.

This Chapter describes the identification, propagation and molecular characterisation of a new set of the nine AHSV reference strains representing one strain for each of the AHSV serotypes. They have been shown to be monotypic using the AHSV type real-time RT-PCR assays. In addition, genome segments encoding the outer capsid proteins (VP2 and VP5) have been sequenced and shown to be identical or very closely related to the respective genome segments of the historical reference strains. These analyses indicate that the new set of the reference strains should have the same antigenic properties as the historical reference strains.

The nine monotypic AHSV strains have been deposited at the ORC, the Pirbright Institute, UK and are now available to researchers and diagnosticians upon request.

Chapter 6: Molecular typing of AHSV and evaluation of intra-serotype variations

6.1: Introduction

Outer-capsid protein VP2 is a primary target for neutralising antibodies and, possibly due to immunological pressure from the hosts' immune system, is the most variable of the AHSV proteins (see Chapter 3) (Bentley *et al.*, 2000; Burrage & Laegreid, 1994; Castillo-Olivares *et al.*, 2011; Martinez-Torrecuadrada & Casal, 1995; Martinez-Torrecuadrada *et al.*, 2001; MartinezTorrecuadrada *et al.*, 1996; Vreede & Huismans, 1994). Variations in the nt sequence of Seg-2 (which codes for VP2) correlate with virus serotype in BTV, EHDV and AHSV (Anthony *et al.*, 2009c; Maan *et al.*, 2007a; Potgieter *et al.*, 2003).

This finding provides a basis for serotype identification of individual virus strains by RT-PCR and/or comparisons of Seg-2 sequences, to a Seg-2 sequence database for established reference strains (Anthony *et al.*, 2009c; Hofmann *et al.*, 2008; Maan *et al.*, 2010a; Maan *et al.*, 2011b; Maan *et al.*, 2008; Maan *et al.*, 2010b; Sailleau *et al.*, 2012). Sequence data have previously been generated for Seg-2 of the AHSV reference strains and were used to analyse the relationship between the different serotypes (Potgieter *et al.*, 2003) (Chapters 3 and 5). Seg-2 from the nine reference strains, is clearly distinguishable, with 55.7-68.2% nt and 49.3-72.3% aa identity respectively between types (Chapter 3).

The development of a Full Length Amplification of cDNA (FLAC) technique (Attoui *et al.*, 2000; Maan *et al.*, 2007b; Potgieter *et al.*, 2009), has facilitated the generation of full length sequences for individual dsRNA genome segments, without a need of

cDNA cloning. This has made it possible to create a sequence database for well documented isolates held in ORC at the Pirbright Institute http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/virus-nos-by-country.htm. The database and virus collection are still expanding to include the whole genomes of more isolates, belonging to different Orbivirus species, from distinct time points and geographic locations.

Phylogenetic analysis of these sequences has provided a greater understanding of the level of nt and aa sequence diversity, within individual genome segments (including Seg-2) and between different strains of these viruses, identifying major 'topotype' groups of BTV and EHDV (Anthony *et al.*, 2009c; Maan *et al.*, 2010b; Maan *et al.*, 2009; Sailleau *et al.*, 2012). The database has also proved to be invaluable for strain identification and molecular epidemiology investigations concerning the origins and movements of individual virus lineages during recent BT outbreaks (Maan *et al.*, 2011b; Maan *et al.*, 2008; Maan *et al.*, 2010b).

Sequence data and phylogenetic analysis of Seg-2 and Seg-10 from AHSV have also been used for molecular investigation of AHS outbreaks in South Africa (Koekemoer *et al.*, 2003; Quan *et al.*, 2008). However, comprehensive studies investigating genetic variation within Seg-2, including multiple isolates from a wider geographic region have not previously been reported.

This Chapter describes the use of an AHSV Seg-2 sequence dataset for the nine reference strains (Chapter 3) to help identify the serotype of vaccine strains and field isolates from a multiple-type outbreak of AHS in Ethiopia during 2010 (Aklilu *et al.*, 2012). Also, the phylogenetic relationships between different isolates of AHSV

serotypes 2, 4, 6, 8 and 9, from different geographical locations within Africa, are investigated.

6.2: Results

6.2.1: Sequence data

Seg-2 sequence data for molecular epidemiological studies was generated from: (a) the nine AHSV reference strains (Chapter 3, section 3.2.3.2); (b) selected AHSV field isolates available at the Orbivirus Reference Collection (ORC) at the Pirbright Institute (Mertens & Attoui, 2011) and (c) publically available AHSV Seg-2 sequences (NCBI database) (Table 6.1).

Although attempts were made to generate full length Seg-2 sequences form the available AHSV isolates using the FLAC technique described in Chapter 2 section 2.6, it was difficult particularly where the isolate contained a mix of different virus strains. Partial sequences of these isolates (marked as "p" for partial sequence) were generated from RT-PCR products as described in Chapter 2 section 2.7.3.

Field isolates accessed from the ORC are annotated with a three letter code indicating the country of the isolate's origin, followed by the year of sample collection and the number of the isolate from that country in that year, e.g. ETH2010/04 stands for a field isolate no 4 from Ethiopia collected in 2010. Similarly, the ORC reference strains are marked with three-letter code for the country of origin, rrah (indicating reference strain) and a number representing serotype of the isolate, e.g. RSArrah/01 stands for AHSV-1 reference strain originated in South Africa. Sequences accessed from NCBI database are annotated with the name of the country of origin and the year

of isolation where available; field strains are indicated by "F" and reference strains by "R". List of the sequences and their details is presented in Table 6.1.

The AHSV Seg-2 sequences were analysed as described in Chapter 2 section 2.11.

6.2.2: Molecular typing of AHSV isolates from Ethiopia in 2010

Full length or partial Seg-2 sequences of three Ethiopian vaccine strains and nine different AHSV field isolates from outbreak in Ethiopia during 2010, were generated for this study (Table 6.1). Two of the vaccine strains (ETH2010/02 and ETH2010/18) and a field strain (ETH2010/23), in each case, generated two distinct Seg-2 sequences. All of the sequences generated were compared to Seg-2 sequence data for reference strains representing each of the nine AHSV serotypes (Chapter 3 section 3.2.3.2).

A clear and close match was observed between each of the field or vaccine strain sequences, and a specific reference strain, in each case identifying the 'type' of the sequence / virus isolate (Figure 6.1). Field isolates from the 2010 AHSV outbreak in Ethiopia were identified as AHSV serotype 2, -4, -6, -8 and -9, as described below in detail.

Table 6.1: List of AHSV isolates used in comparative Seg-2 sequencing studies.

Virus serotype	Isolate designation*	Country of origin	Passage history**	Year of Isolation	Seg-2 length (bp)	NCBI database reference†
AHSV-1	RSArrah/01	South Africa	MB3/BHK7	1962	3218	
AHSV-2	RSArrah/02	South Africa	MB3/BHK7	1961	3218	
	SEN2007/02	Senegal	MB1/BHK3	2007	3218	
	SEN2007/04	Senegal	MB1/BHK3	2007	3218	
	SEN2007/05	Senegal	MB1/BHK3	2007	3218	
	ETH2010/01	Ethiopia	KC1	2010	3218	
	ETH2010/23p	Ethiopia	KC1	2010	500	
	ETH2010/02p	Ethiopia/vaccine strain	V1	2010	927	
	GHA2010/01p	Ghana	KC1	2010	927	
	Nigeria 2007	Nigeria/field strain	blood	2007	3218	FJ196585
AHSV-3	RSArrah/03	South Africa	MB3/BHK5	1963	3221	
AHSV-4	SPArrah/04	Spain	BHK3	1987	3229	
	SPA1987/01	Spain	BHK1	1987	3229	
	KEN2007/01	Kenya	V3	2007	3229	
	ETH2010/05	Ethiopia	KC1	2010	3229	
	ETH2010/07	Ethiopia	KC1	2010	3229	
	ETH2010/08	Ethiopia/vaccine strain	V1	2010	3229	
	S. Africa (F1)	South Africa/ field	-	Recent	3229	EU046574
	S. Africa (F2)	South Africa/Field	-	Recent	3229	DQ868773
	Iran	Iran/vaccine strain	-	-	3229	D26570
AHSV-5	RSArrah/05	South Africa	MB3/BHK6	1962	3217	
AHSV-6	RSArrah/06	South Africa	BHK3	1963	3202	
	ETH2010/19	Ethiopia	KC1	2010	3202	
	S. Africa (F)	South Africa/field strain	-	Recent	3203	DQ868774
	S. Africa (R)	South Africa/ref strain	-	1963	3203	AF021235
AHSV-7	KENrrah/07	Kenya	MB3/BHK4	1962	3221	
	SEN2007/06	Senegal		2007	3221	
	S. Africa (F1)	South Africa	MB1/BHK3	1998	786	AY159943
	S. Africa (F2)	South Africa	MB1/BHK3	1998	786	AY159947
AHSV-8	RSArrah/08	South Africa	BHK1	1962	3217	
	ETH2010/10	Ethiopia	KC1	2010	3217	
	ETH2010/11	Ethiopia	KC1	2010	3217	
	S. Africa (F)	South Africa	-	Recent	3217	DQ868775
AHSV-9	PAKrrah/09	Pakistan	MB3/BHK7	1961	3202	
	ETH2010/16	Ethiopia	KC1	2010	3202	
	ETH2010/17	Ethiopia	V1	2010	3202	
	ETH2010/18p	Ethiopia-vaccine	V1	2010	854	
	ETH2010/23	Ethiopia	KC1	2010	3202	
	SEN1998/01p	Senegal	V1/BHK1	1998	1654	
	GAM2009/03	Gambia	KC2	2009	3202	
	KEN2006/01p	Kenya	V1/E1/BHK	2006	1654	
	SENvvvv/09	Senegal	-	-	3202	
	S. Africa (F)	South Africa/field strain	-	Recent	3202	DQ868775

(*)Full details on isolates from ORC isolates are available at http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/AHSV-isolates.htm (Mertens & Attoui, 2011). (**)MB: Mouse brain, BHK: Bovine hamster kidney cell line, V: Vero cells, KC: Cells derived from *Culicoides sonorensis*, E: hens eggs embryo. (†) Published Seg-2 sequences used for comparisons; Seg-2 data from all other isolates were generated during these studies. (-) Data not available. (Recent) sequences submitted as 'recent field isolates' in 2006.

AHSV-2: Three isolates, ETH2010/01, ETH2010/02 and ETH2010/23, were identified as AHSV-2. Comparisons of Seg-2 from two field isolates (ETH2010/01 – full length, ETH2010/23, partial sequence) and a vaccine stain (ETH2010/02, partial sequence), showed 96-97% / 98.4-98.8% - nt/aa identity to Seg-2 of the AHSV-2 reference strain (RSArrah/02) (Figure 6.1, Table 6.2). This very high level of identity identifies the isolates as serotype 2. The three Ethiopian field isolates, which show >99.6% nt identity, have identical VP2 proteins (Table 6.2).

Two isolates, ETH2010/23 (field strain) and ETH2010/01 (vaccine strain), generated mixed Seg-2 sequences, showing that they each contain a mixed virus population belonging to different serotypes. The field isolate ETH2010/23, generated a full length Seg-2 sequence of AHSV-9, as well as partial Seg-2 sequence of AHSV-2 (1051-1551 nt) showing 97% and 96.4% nt identity to the AHSV-9 and AHSV-2 reference strains respectively (Table 6.2). Two Seg-2 sequences from the AHSV-2 vaccine strain ETH2010/02, showed nt identity of 96.4% (over 479-1517 bp) and 99.7% (3221 bp which is full length) to the reference strains of AHSV-2 and AHSV-3 respectively, indicating that it also represents a mix of these two AHSV serotypes.

AHSV-4: Two field isolates (ETH2010/05 and ETH2010/07) showed a very close relationship to each other (99.6% nt, 99.8% aa identity) and to the reference strain (SPArrah/04) (97.5% nt and 98.7% aa identity) (Table 6.2; Figure 6.1), identifying them as AHSV-4. However, Seg-2 of these Ethiopian field isolates (ETH2010/05 and ETH2010/07) are more distantly related to the Seg-2 of the Ethiopian AHSV-4 vaccine strain (ETH2010/08) (91% nt and 96.1% aa identity), demonstrating that they are not derived from this origin. The Ethiopian AHSV-4 vaccine strain and the AHSV-4 reference strain from Spain (SPArrah/04) were also more distantly related (91.7% nt and 96.7% aa identity) (Table 6.2). However, the Ethiopian vaccine

(ETH2010/08) was very closely related (99.8%) to the sequence published for an Iranian AHSV-4 vaccine (Sakamoto *et al.*, 1994a) (Table 6.2; Figure 6.2b).

AHSV-6: Phlyogenetic analysis of the full length Seg-2 sequence generated from ETH2010/19, grouped this isolate with the reference strain of AHSV-6 (RSArrah/06) (showing 91.5% nt and 96% aa identity), identifying it as an isolate of AHSV-6 (Figure 6.1, Table 6.3).

AHSV-8: The full length sequences of Seg-2 from two field isolates (ETH2010/10 and ETH2010/11) showed 97.5% nt and 98.1/98.2% aa identity, compared to the AHSV-8 reference strain (RSA2010/08) identifying these isolates as AHSV-8 (Figure 6.1, Table 6.3). Seg-2s from these field isolates showed a very high level of nucleotide (99.7%) and amino acid (99.8%) identity, consistent with being derived from the same lineage /outbreak.

AHSV-9: Full length Seg-2 sequences from three Ethiopian field isolates (ETH2010/16, ETH2010/17 and ETH2010/23) and a partial Seg-2 sequence from the Ethiopian AHSV-9 vaccine strain (ETH2010/18) (531-1887 bp) showed high level nt (96.7, 96.6, 97.2, 97% respectively) and aa (98.3, 97.3, 98.3, 99.6% respectively) identity to that of the AHSV-9 reference strain (PAKrrah/09) (Table 6.1 and 6.3), identifying them as AHSV-9. The Ethiopian AHSV-9 vaccine strain (ETH2010/18) also generated a Seg-2 sequence that showed 99.4% nt identity with Seg-2 of the AHSV-5 reference strain (data not shown).

6.2.2: Intra-serotype variation of AHSV serotypes 2, 4, 6, 7, 8 and 9.

Full length or partial Seg-2 sequences were compared for field, vaccine and reference strains of AHSV serotype 2, 4, 6, 7, 8 and 9 from different geographical locations in Africa (Table 6.1). The levels of nt and aa identity were calculated (Table 6.2) providing evidence of intra-serotypic variations in the different serotypes as described below.

AHSV-2: Nine different Seg-2s nt and aa sequences were compared for AHSV-2 (Table 6.1). These include, the Pirbright Institute AHSV-2 reference strain (RSArrah/02), Ethiopian vaccine strain (ETH2010/02) and AHSV-2 field isolates originated from recent outbreaks (SEN2007/02, SEN2007/04, SEN2007/05, GHA2010/01, ETH2010/01, ETH2010/023, Nigeria 2007 [Ac. No. FJ196585]) (Fasina *et al.*, 2008; ProMED-mail, 2007a, 2010) (Table 6.1).

Full length Seg-2 was compared for most isolates but only partial sequences were generated from GHA2010/01 (591-1890 bp), ETH2010/02 (479-1517 bp) and ETH2010/23 (479-1517 bp) (Table 1). All of the isolates analysed were closely related, showing more than 96% nt and 98 % AA identity (Table 6.2). Despite these close overall relationships, two clusters were identified: cluster A, populated by isolates from the recent outbreaks in central Africa (Senegal, Nigeria, Ghana and Ethiopia) (with >99% nt and aa identity), and cluster B containing the AHSV-2 reference strain (RSArrah/02) which was originally isolated in South Africa in 1961 (Figure 6.2a). The field isolates shared 96-97.2% nt and 98.4-99% aa identity with the AHSV-2 reference strain (Table 6.2).



Figure 6.1: Phylogenetic tree for Seg-2 nucleotide sequences of the Ethiopian AHSV vaccines and field strains of from 2010 outbreak.

The tree was constructed by neighbour-joining methods using distance matrices, generated by the p-distance determination algorithm in MEGA version 5 software (500 bootstrap replicates) (Tamura *et al.*, 2011).

Table 6.2: Percentage identities for Seg-2/VP2 from isolates of AHSV-2 and 4.

	RSArrah/02	SEN2007/02	SEN2007/04	SEN2007/05	ETH2010/01	ETH2010/23p	ETH2010/02p	GHA2010/01p	Nigeria 2007	SPArrah/04	SPA1987/01	KEN2007/01	ETH2010/05	ETH2010/07	ETH2010/08	S.Africa (F1)	S.Africa (F2)	Iran
RSArrah/02		98.7	98.9	99.0	98.7	98.8	98.4	98.4	99.0	52.3	52.2	52.1	52.1	52.0	51.1	51.9	52.0	51.1
SEN2007/02	97.0		99.6	99.7	99.2	100	99.7	99.7	99.7	52.2	52.1	52.1	52.1	52.0	51.1	51.8	51.9	51.1
SEN2007/04	97.1	99.8		99.9	99.4	99.4	99.7	99.7	99.9	52.1	52.0	52.0	52.0	52.0	51.1	51.8	52.0	51.1
SEN2007/05	97.2	99.8	99.9		99.5	100	100	100	100	52.2	52.1	52.1	52.1	52.0	51.1	51.8	51.9	51.1
ETH2010/01	97.0	99.3	99.3	99.4		100	100	100	99.5	52.1	52.0	52.0	52.0	51.9	51.1	51.7	51.8	51.1
ETH2010/23p	96.4	99.6	99.4	99.6	99.6		100	100	100	53.3	53.3	53.3	53.3	53.3	53.3	52.7	52.1	53.3
ETH2010/02p	96.0	99.4	99.4	99.5	**100**	99.6		100	100	48.4	48.4	48.4	48.4	48.4	47.1	47.7	47.4	47.1
GHA2010/01p	96.4	99.6	99.6	99.7	99.1	99.4	99.1		100	48.4	48.4	48.4	48.4	48.4	47.1	47.7	47.4	47.1
Nigeria 2007	97.2	99.8	99.9	99.9	99.4	99.6	99.5	99.7		52.2	52.1	52.1	52.1	52.0	51.1	51.8	51.9	51.1
SPArrah/04	56.8	56.6	56.6	56.6	56.4	60.3	56.4	57.1	56.6		99.9	98.8	98.7	98.7	96.7	99.5	99.5	96.3
SPA1987/01	56.8	56.6	56.6	56.6	56.5	60.5	56.5	57.2	56.7	99.9		98.7	98.6	98.6	96.6	99.4	99.4	96.2
KEN2007/01	56.6	56.6	56.6	56.6	56.3	60.3	56.7	57.5	56.6	97.6	97.5		99.9	99.9	96.2	98.3	98.3	95.8
ETH2010/05	56.7	56.6	56.6	56.6	56.4	60.3	56.8	57.6	56.6	97.5	97.4	99.8		99.8	96.1	98.2	98.2	95.8
ETH2010/07	56.6	56.5	56.5	56.5	56.3	60.1	56.7	57.5	56.6	97.5	97.4	99.8	99.6		96.1	98.2	98.2	95.8
ETH2010/08	56.3	56.6	56.6	56.6	56.6	62.1	56.8	57.0	56.6	91.7	91.6	91.0	90.9	91.0		96.2	96.2	99.4
S.Africa (F1)	56.7	56.5	56.5	56.5	56.3	60.1	56.2	56.9	56.5	99.8	99.7	97.4	97.3	97.3	91.6		99.1	95.8
S.Africa (F2)	56.7	56.5	56.5	56.5	56.4	59.9	55.9	56.7	56.6	99.8	99.7	97.4	97.2	97.2	91.6	99.6		95.8
Iran	56.1	56.4	56.5	56.4	56.5	61.7	56.6	56.9	56.5	91.6	91.5	90.9	90.8	90.8	99.8	91.4	91.5	

Percentage identities for AHSV-2 are highlighted in blue and AHSV-4 in red with lighter colours and darker colours indicating comparisons of nt and aa sequences respectively. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

Table 6.3: Percentage identities for Seg-2/VP2 from isolates of AHSV- 6, 7, 8 and 9.

	RSArrah/06	ETH2010/19	S.Africa (F)	S.Africa (R)	KENrrah/01	SEN2007/06	S.Africa (F1)p	S.Africa (F2)p	RSArrah/08	ETH2010/10	ETH2010/11	S.Africa (F)	PAKrrah/09	ETH2010/16	ETH2010/17	ETH2010/18p	ETH2010/23	SEN1998/01p	GAM2009/03	KEN2006/01p	SENvvvv/09	S.Africa (F)
RSArrah/06		96.0	96.2	98.8	51.5	51.5	51.1	50.8	52.1	52.2	52.3	52.2	62.5	62.8	62.4	64.9	62.8	64.0	62.3	63.9	62.1	62.0
ETH2010/19	91.5		97.0	94.7	50.5	50.5	49.6	49.2	52.0	52.1	52.1	52.3	62.1	62.4	62.1	64.1	62.4	63.7	62.0	63.9	61.7	61.9
S.Africa (F)	92.3	93.7		95.0	50.9	50.9	50.4	50.0	52.0	52.1	52.1	52.1	62.6	62.9	62.5	64.8	62.9	64.4	62.5	64.4	62.2	62.5
S.Africa (ref)	99.7	91.1	91.9		50.8	50.8	50.0	49.6	51.5	51.7	51.8	51.7	61.7	62.0	61.6	64.2	62.0	63.5	61.5	63.3	61.3	61.3
KENrrah/01	57.4	56.9	57.0	57.3		97.7	98.9	98.9	51.2	51.2	51.2	51.4	51.8	51.8	51.5	46.0	51.8	46.7	51.4	46.4	51.5	51.2
SEN2007/06	56.9	56.1	56.6	56.7	96.6		98.9	98.9	51.6	51.6	51.6	52.5	51.5	51.5	51.2	45.8	51.5	46.7	51.1	46.4	51.2	51.0
S.Africa (F1)p	59.5	58.9	60.0	59.7	97.5	95.3		98.5	51.3	51.3	51.3	52.1	52.3	52.3	53.1	51.9	52.3	53.1	53.1	51.9	51.5	51.9
S.Africa (F2)p	58.6	58.5	58.9	58.8	97.3	96.9	95.5		51.7	51.3	51.3	51.8	52.3	52.3	52.3	51.9	52.3	52.3	52.3	51.9	51.5	51.9
RSArrah/08	57.4	57.3	57.3	57.1	56.5	56.5	56.3	56.8		98.2	98.1	98.9	53.1	53.0	52.5	50.4	53.0	51.3	52.5	51.5	52.4	53.2
ETH2010/10	57.3	57.4	57.2	57.1	56.7	56.7	55.9	56.6	97.5		99.8	98.1	52.8	52.9	52.6	50.2	52.9	51.5	52.6	51.1	52.3	52.8
ETH2010/11	57.3	57.3	57.3	57.1	56.8	56.8	56.0	56.8	97.5	99.7		98.2	52.9	53.0	52.6	50.2	53.0	51.5	52.7	51.2	52.4	52.9
S.Africa (F)	57.7	57.5	57.7	57.4	56.9	58.2	57.4	56.9	98.1	97.2	97.2		53.4	53.3	53.3	52.8	52.8	52.7	53.5	51.8	52.0	51.1
PAKrrah/09	63.0	62.9	62.9	62.7	56.5	55.9	57.5	57.1	58.9	59.0	59.0	59.3		98.3	97.3	99.6	98.3	97.5	97.4	95.5	99.1	94.9
ETH2010/16	63.0	63.0	63.1	62.8	56.6	56.0	57.6	57.2	58.5	58.8	58.8	57.7	96.9		97.0	97.1	100	96.7	97.1	94.7	97.4	94.5
ETH2010/17	63.1	62.7	62.7	62.9	56.1	55.6	57.5	57.1	58.3	58.5	58.5	58.9	96.7	95.4		96.3	97.0	99.8	99.4	94.4	96.7	93.5
ETH2010/18p	65.1	65.4	65.5	64.9	54.3	53.9	57.1	56.7	57.0	57.0	57.1	59.0	99.6	97.1	96.3		97.1	96.7	96.3	94.8	99.8	94.5
ETH2010/23	63.0	63.0	63.1	62.7	56.4	55.8	57.6	57.2	58.5	58.7	58.7	58.6	97.0	99.8	95.3	97.1		96.7	97.1	94.7	97.4	94.5
SEN1998/01p	64.1	64.3	64.7	64.0	54.1	53.3	57.3	56.9	57.0	57.0	57.0	57.2	97.2	96.0	99.3	96.9	96.0		99.6	94.6	96.9	94.2
GAM2009/03	63.2	62.8	62.8	62.9	56.3	55.8	57.3	56.9	58.3	58.4	58.4	58.5	96.9	95.5	99.3	96.3	95.5	99.2		94.2	96.8	93.7
KEN2006/01p	63.3	63.5	63.6	63.4	55.1	54.4	58.5	57.8	58.2	57.8	57.8	58.5	90.9	90.1	89.7	90.4	90.0	89.8	89.5		94.9	98.5
SENvvvv/09	62.9	62.7	62.7	62.6	56.3	55.7	56.9	56.6	58.7	58.8	58.8	59.1	99.4	96.5	96.4	99.9	96.6	96.9	96.6	90.6		94.0
S.Africa (F)	62.5	61.8	62.0	62.4	56.9	56.4	57.6	57.2	58.5	58.3	58.3	58.6	90.0	88.8	88.9	89.8	88.9	89.6	88.9	96.7	89.7	

Percentage identities of AHSV-6 are highlighted in green, AHSV-7 in purple, AHSV-8 in orange and AHSV-9 in olive with lighter colour indicating comparisons of nucleotide and darker amino acid sequences. Analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

AHSV-4: The Seg-2/VP2 sequences were compared for nine different field, vaccine and reference strains of AHSV-4 (Table 6.1). The field isolates originated from the AHSV-4 outbreak in Spain in 1987 (SPArrah/04, SPA1987/01), the 2007 AHSV outbreak in Kenya (KEN2007/01), multiple serotype outbreaks in Ethiopia during 2010 (ETH2010/05, ETH2010/07) and recent field strains from South Africa (Ac. No. EU046574, DQ868773). These analyses also included the Ethiopian and the Iranian AHSV-4 vaccines (ETH2010/08 and Ac. No. D26570) (Table 6.1).

The overall identity-level of Seg-2/VP2 from the isolates of AHSV-4 that were compared, is >90.9% nt and >95.8% aa (Table 6.2). These isolates group into three distinctive clusters: Cluster A, with >99% nt and aa identity, contains the Spanish strains SPArrah/04 and SPA1987/01, and South African field isolates (Ac. No. EU046574, DQ868773); Cluster B (>99% nt and aa identity between isolates) includes the Kenyan strain KEN2007/01, and the Ethiopian field isolates ETH2010/05 and ETH2010/07; Cluster C (with 99.8% nt and aa identity) contains the Ethiopian and the Iranian AHSV-4 vaccine strains (ETH2010/08 and Ac. No. D26570) (Figure 6.2b).

Comparisons of the Seg-2 sequences between these clusters showed pronounced differences. The percentage identities between cluster A and B (both, grouping Seg-2 of field isolates) are >97% nt and >98% aa. However both clusters A and B share only >90% nt and >95% aa identities with cluster C (Table 6.2).

AHSV-6: Full length sequences of Seg-2 were compared from four strains of AHSV-6, including ETH2010/19; RSArrah/06 (the reference strain from the ORC); the South African reference strain (published sequence Ac No. AF021235) and a South African field isolate (published sequence Ac No. DQ868774) (Table 6.1). The two AHSV-6 reference strains from the ORC (RSArrah/06) and South Africa (Ac. No. AF021235)

grouped together and showed high level of identity (>99% nt and 98% aa) confirming that they are derived from the same source. The field strains from the 2010 outbreak in Ethiopia (ETH2010/19) and a recent field strain from South Africa (Ac. No. DQ868774) were more distantly related to the reference strains (>91% nt and >94% aa identity) or to each other (93.7% nt and 97% aa identity) (Table 6.3, Figure 6.2c). This indicates that the recent circulation of this AHSV type in Ethiopia and South Africa are not due to geographic extensions of the same outbreak.

AHSV-7: Full length Seg-2 sequences of the AHSV-7 reference strain (KENrrah/07), a field strain from Senegal (SEN2007/06), and partial Seg-2 sequences from South African field strains (Ac. No. AY159943 and AY159947) (Koekemoer *et al.*, 2003) were compared (Table 6.1). Seg-2 sequence of all AHSV-7 strains investigated were almost equally distant to each other with >95% nt and >97% aa identity (Table 6.3) forming four separate clades (Figure 6.3d).

AHSV-8: Full length sequences of Seg-2 were compared for four AHSV-8 strains, including: two isolates from Ethiopian outbreaks during 2010 (ETH2010/10, ETH2010/11), a recent fields strain from South Africa (Ac. No. DQ868775) and the AHSV-8 reference strain (RSArrah/08) (Table 6.1). The two Ethiopian isolates grouped together with 99.7% nt identity, indicating their common recent origin, but were more distantly related to the South African field strain (Ac. No. DQ868775) with 97.2% nt and 98.5% aa identity or the AHSV-8 reference strain with 97.5% nt and 99% aa identity (Table 6.3, Figure 6.3e).

AHSV-9: The Seg-2 sequences were compared from ten different strains of AHSV-9 (Table 6.1). These include full length sequences of: the monovalent vaccine from Senegal (SENvvvv/09), the AHSV-9 reference strain (PAKrrah/09), a recent field strains from

South Africa (Ac. No. DQ868775), ETH2010/16, ETH2010/17, ETH2010/23, and GAM2009/03; as well as partial Seg-2 sequences from the Ethiopian AHSV-9 vaccine strain (ETH2010/18) (531-1897 bp), field isolates from Kenya (KEN2006/01) (616-2268 bp) and Senegal (SEN1998/01) (616-2268 bp) (Oura *et al.*, 2011; Sailleau *et al.*, 1997b).

The overall identity level between these isolates was calculated > 88% nt and >93% aa (Table 6.3). They are divided into four distinct clusters (A, B, C and D) (Figure 6.2f). Seg-2 sequences of the AHSV-9 vaccine strains (SENvvvv/09; ETH2010/18) and reference strains (PAKrrah/09) grouped in Cluster A. They were found to be very closely related with >99% nt and aa identity indicating that they were recently derived from a common source (Table 6.3). Cluster B contains two virus isolates form the 2010 outbreak in Ethiopia (ETH2010/16 and ETH2010/23). The nucleotide sequences in Cluster B were almost identical over the full length of Seg-2, with just one synonymous difference in ETH2010/16. Cluster C includes field isolates from Ethiopia (ETH2010/17), Senegal (SEN1998/01) and Gambia (GAM2010/03). The isolates in Cluster C are also closely related to each other, with >99% nt and aa identity (Table 6.3). Cluster D includes two Seg-2 sequences, a South African field strain (Ac. No. DQ868776) and Kenyan isolate from 2006 (KEN2006/01), which share 96.7% nt and 98.5%AA identity (Table 6.3).

Clusters A, B and C are more closely related to each other (>95 to 96.5% nt identities between the clusters) than to cluster D, with which they share only 89.7, 88.8 and 88.9% nt identities, respectively (Table 6.3, Figure 6.3f).

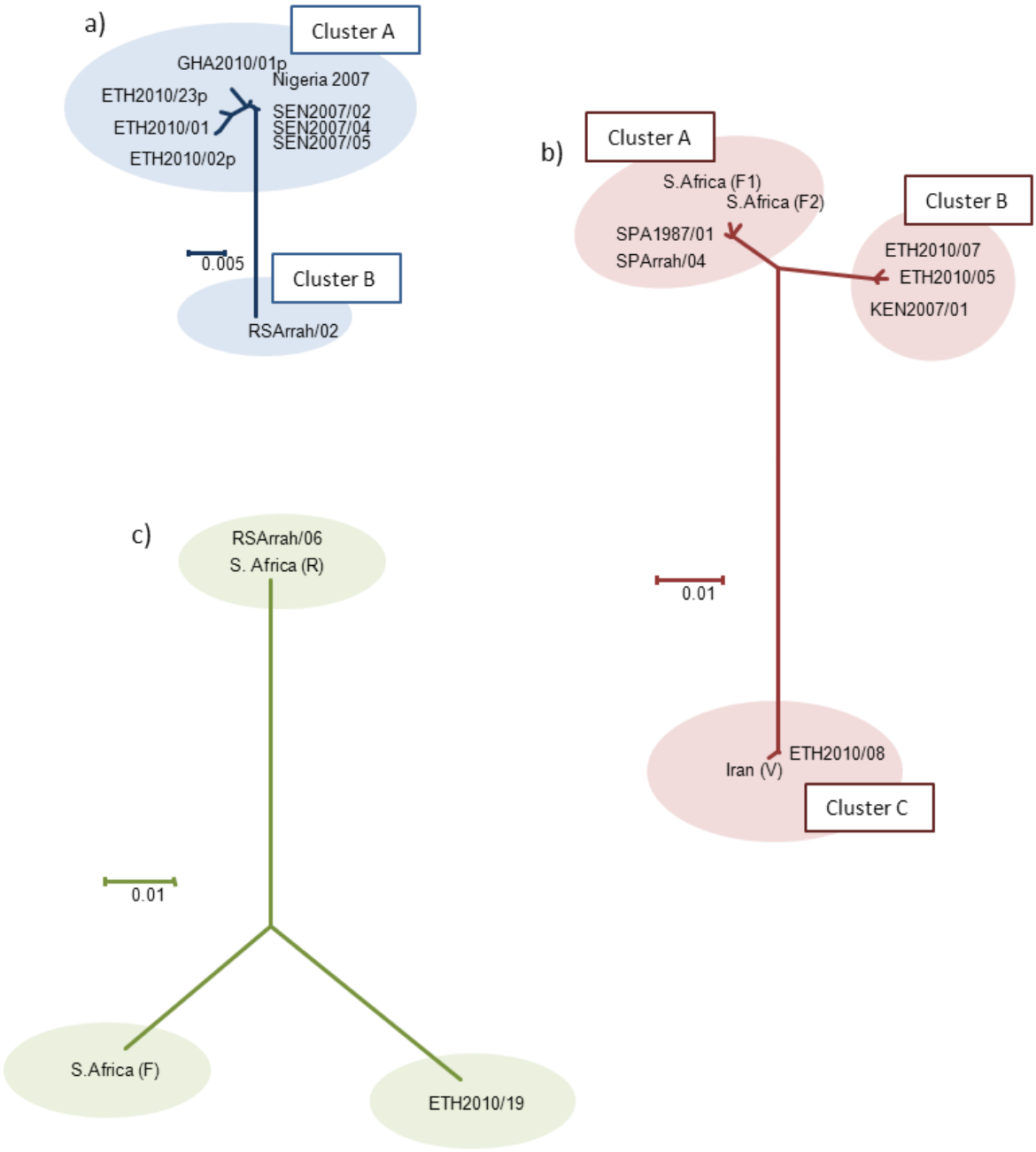


Figure 6.2: Neighbour-joining phylogenetic trees showing Seg-2 relationships within AHSV serotypes 2, 4 and 6.

Different trees illustrating the relationship between Seg-2 nt sequences within a) AHSV-2, b) AHSV-4, c) AHSV-6. The trees were constructed using Neighbour-joining distance matrices, generated by the p-distance determination algorithm in MEGA version 5 software (Tamura *et al.*, 2011).

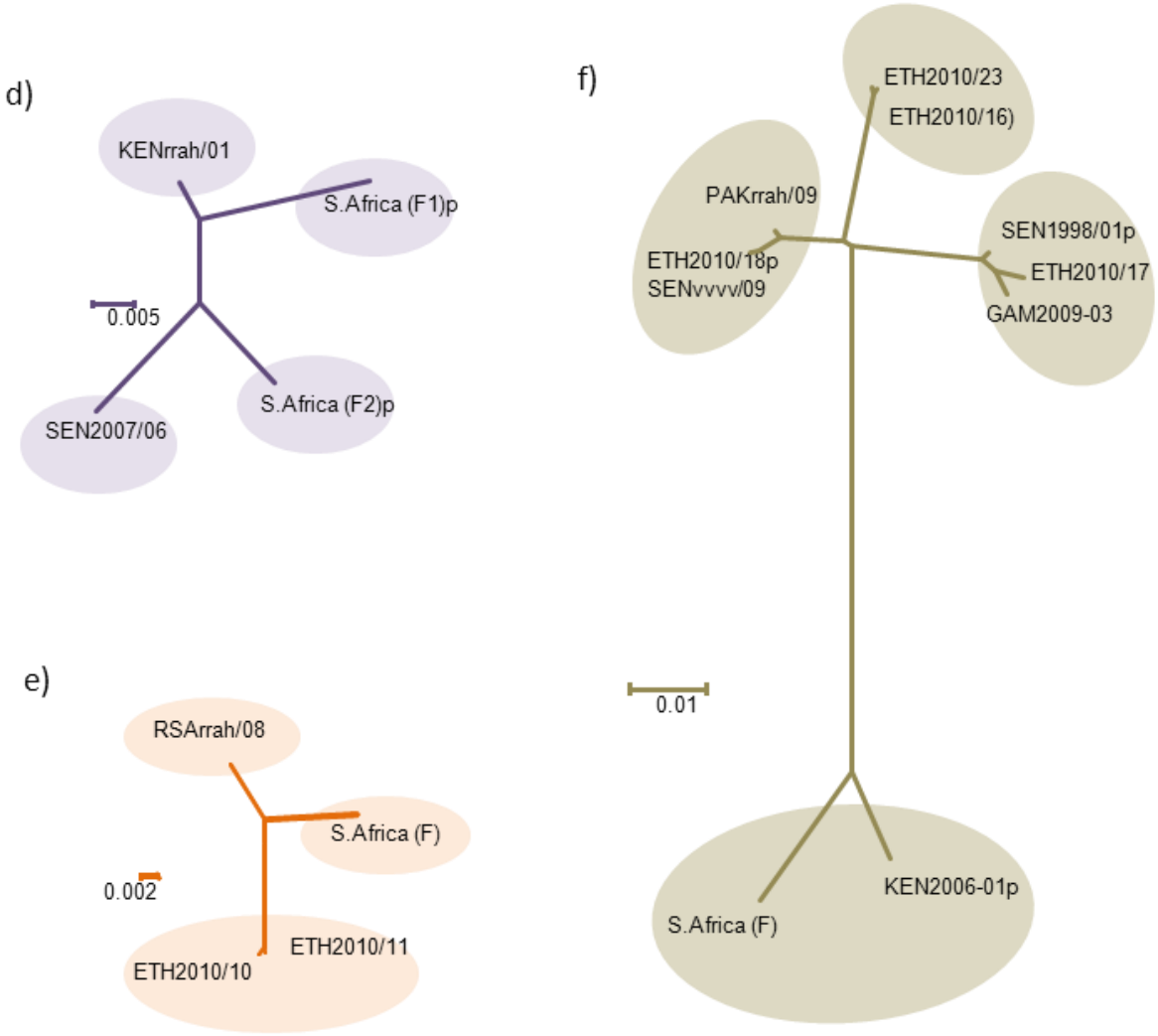


Figure 6.3: Neighbour-joining phylogenetic trees showing Seg-2 relationships within AHSV serotypes 7, 8 and 9.

Different trees illustrating the relationship between Seg-2 nt sequences within d) AHSV-7, e) AHSV-8 and f) AHSV-9. The trees were constructed using Neighbour-joining distance matrices, generated by the p-distance determination algorithm in MEGA version 5 software (Tamura *et al.*, 2011).

6.3: Discussion

Five different serotypes of AHSV (AHSV 2, 4, 6, 8 and 9) were identified using real-time RT-PCR assays targeting Seg-2 (see Chapter 4), in isolates from wide spread outbreaks of AHS in Ethiopia during 2010. To validate these findings, Seg-2 nucleotide sequences from representative isolates of each serotype detected, were compared to the historical reference strains. In each case a close match was detected with the reference strains of the relevant serotype, confirming strain identification.

This is the first time that an AHSV outbreak involving 5 out of the 9 serotypes of AHSV has been reported outside South Africa (ProMED-mail, 2009). Moreover, AHSV serotypes 6 and 8 were detected in central Africa for the first time.

Historically, AHS in central Africa has been caused by serotype 9, with the remaining isolates of AHSV circulating in the south of the continent (Coetzer & Guthrie, 2004). The 1987-1991 outbreak of AHSV-4 in Spain and Portugal started when AHSV-4 infected zebra were imported to Madrid from Namibia (Lubroth, 1988; Rodriguez *et al.*, 1992; Sanchez-Vizcaino, 2004) (see also Chapter 1 section 1.7.1).

Two serotypes of AHSV, AHSV-6 and AHSV-8, which had not previously been reported in central Africa, were identified during the 2010 outbreak in Ethiopia. The previous origin of these strains is not clear. However, the recent expansion of AHSV-2 in central Africa (see Chapter 1 section 1.7.1), suggests that the other serotypes detected in Ethiopia could become established and could affect equids in central and west African countries.

In addition to AHSV-9, several additional serotypes of AHSV have been detected in central Africa Since 2007, including AHSV-2, 4, 6, 7, and 8 (see Chapter 1 section 1.7.1). This suggests that conditions have been particularly favourable for the emergence of these serotypes into new areas. However, it is also possible that these types have been

circulating in the area for some considerable time but had not previously been identified, due to a shortage of appropriate methods and/or reagents. The recent development of novel typing assays for AHSV (see Chapter 1, section 1.8 and Chapter 4) have improved sample throughput and the sensitivity of serotype identification tests, making it possible to screen more samples, and improving detection. However, there is also a need for individual cases of the disease to be reported for full diagnosis, so that samples can be collected, along with full epidemiological data (including date, location, host species and clinical signs) to provide a better understand the molecular epidemiology of the virus.

The AHS outbreaks in Ethiopia during 2010, involved 5 different serotypes of the virus, which are able to re-assort, creating new virus strains, that may have different and possibly even new biological properties (O'Hara *et al.*, 1998). Multiple serotype outbreaks of AHSV and BTV have been shown to result in emergence of re-assorted viruses (Batten *et al.*, 2008b; von Teichman & Smit, 2008). One of the Ethiopian field isolates (ETH2010/23) was found to contain Seg-2 sequences of two different serotypes AHSV-2 and AHSV-9. Full genome analysis of this isolate or other mixed isolates (using next generation sequencing technologies) would help to track the processes of genome segment reassortment and the subsequent selection of possible progeny strains.

AHSV Seg-2 sequence data from recent outbreaks in central Africa (accessed from the ORC, the Pirbright Institute (Mertens & Attoui, 2011)) and other publicly available Seg-2 sequence data were used to determine the level of variation within the nt sequence of Seg-2 of AHSV serotypes 2, 4, 6, 7, 8 and 9. The level of intra-serotypic variation within Seg-2 of these AHSV serotypes is reported here for the first time.

Based on the sequence data available, generally high levels of Seg-2 nt identity were identified within each of the AHSV serotypes investigated (AHSV-2: 96.4-100%, AHSV-

4: 90.8-99.9%, AHSV-6: 91.1-99.7%, AHSV-7: 95.3-96.9%, AHSV-8: 97.5-99.7%, AHSV-9: 88.8-99.9%). These levels are significantly higher than detected between different topotypes within individual serotypes of BTV or EHDV (>49% and >45% nt identity respectively) (Anthony *et al.*, 2009c; Maan *et al.*, 2007a) again suggesting that AHSV does not exist as distinct topotypes, possibly due to its geographic distribution restricted to a single continent. Despite, the close Seg-2 phylogenetic relationship of AHSV within individual serotypes, distinct groupings of isolates was observed.

Phylogenetic comparisons of Seg-2 from the AHSV-2 field strains investigated grouped closely together (>99% nucleotide identity) indicating a recent common ancestry. However, they were more distantly related to the South African reference strain which formed a separate group (Figure 6.2a). All of the Seg-2 sequences compared were obtained from isolates collected in central Africa between 2007 and 2010 (see Chapter 1 section 1.7.1). No recent virus isolates or sequence data were available for this study from the South of the continent, where AHSV-2 is endemic. However, a study of phylogenetic relationships between partial AHSV-2 Seg-2 sequences from the 2007 outbreaks in west Africa (Nigeria and Senegal) and of viruses circulating in southern Africa during 2006 (South Africa and Botswana) showed that they were very closely related (Fasina *et al.*, 2008). This finding supports a suggestion made by Fasina et al., (2008) that the outbreak in Nigeria during 2007, was caused by illegal importation of an infected horse into the country. A third cluster of AHSV-2 Seg-2 was also described which groups two field isolates from KawZulu-Natal, South Africa, in addition to the two clusters (cluster A and B) identified in the present investigation (Figure 6.2a) (Fasina *et al.*, 2008). Sequence data of the KawZulu-Natal isolates is not available and therefore could not be used in the present study.

Only a limited number of AHSV isolates / Seg-2 sequences of serotypes 4, 6, 7 and 8 were available for analysis in this study, but a clear phylogenetic pattern could be observed. Seg-2 sequences of the recent South African isolates grouped separately to the sequences obtained from isolates from recent outbreaks in central Africa. Furthermore, all field isolates were found to be only distantly related to the reference/vaccine strains. This suggests that the isolates of AHSV-4, 6, 7 and 8 from South of Africa (Ac. No. EU046574, DQ868773, DQ868774, DQ868775, AY159943 and AY159947) originated from a different origin / lineage / circulation of AHSV, from those in central Africa. However, most of these viruses were also isolated on significantly different dates (up to 49 years apart), as well as from different geographic locations (Table 6.1). This time differences may have allowed them to accumulate multiple different changes / point mutations in their respective nucleotide sequences.

Interestingly, the IAH AHSV-4 reference strain from the 1987 AHSV-4 outbreak in Spain was reported to originate from AHSV-4 infected zebra imported into a Safari Park near Madrid (Lubroth, 1988; Rodriguez *et al.*, 1992; Sanchez-Vizcaino, 2004). Phylogenetic analysis of AHSV-4 Seg-2 revealed very close relationship (>99% nucleotide identity) between the Spanish isolates (SPA1987/01 and SPArrah/04) and field isolates from South Africa (Ac. No. EU046574 and DQ868773) indicating a recent common ancestry and supporting epidemiological information on the origin of the AHSV-4 outbreak in Spain in 1987.

A previous study using partial Seg-2 sequences from AHSV-7 outbreaks in South Africa during 1999, and comparisons to historical strains showed that the isolates grouped into two separate clusters (>95.5% nt identity between clusters) but separately from the AHSV-7 reference strain (> 96.8 and 97.2 % nt identity) (Koekemoer *et al.*, 2003). The two most distinctive sequences from the study were also included in the study described

here (Ac Nos. AY159943, AY159947), showing that Seg-2 of the isolates from west Africa (SEN2007/06) group independently from those in South Africa or the reference strains (Figure 6.3d).

Seg-2 sequences of AHSV-9 grouped into four separate and distinctive clusters: A, B, C and D. Cluster A includes the AHSV-9 reference strain that originated from the outbreak in Pakistan during 1959 (PAKrrah/09) and the Senegalese (SENvvvv/09) and the Ethiopian (ETH2010/18) vaccine strains derived from it (Figure 6.3f). Interestingly, Cluster C includes strains from different countries across central Africa (Senegal, Gambia and Ethiopia), which show > 99% nt identity in Seg-2. In contrast the field strains from the 2010 outbreaks in Ethiopia grouped in two distinctive clusters (Cluster B and C) suggesting that the outbreaks could have originated from at least, two different origins / lineages, one of which is distinct from the other central African isolates of AHSV- 9. It is possible that the lineage of type 9 that was unique to Ethiopia (Cluster B) has been present as a distinct circulation in the region for some considerable time.

Isolates grouped within clusters A, B and C are very closely related (> 99% nt identity), indicating a recent common ancestry but clear phylogenic distance > 95.3 to 96.5% nucleotide identity was estimated between the clusters. Two isolates (KEN2006/01 and South African field strain - DQ868776) grouped within cluster D, distantly to clusters A, B and C, but the phylogenetic distance of 96.7% nt identity between these isolates is almost in the range of the nucleotide distances between clusters A, B, C. This suggests that both of the isolates forming cluster D, may become parts of separate phylogenetic groupings after wider phylogenetic study.

6.3.1: Summary

Representative AHSV isolates from different geographical location in Africa were used to examine the levels of nucleotide variation within Seg-2 of AHSV serotypes 2, 4, 6, 7, 8 and 9. This study shows / confirms that:

- comparisons of Seg-2 sequences from individual AHSV isolates, to a Seg-2 dataset for the nine AHSV reference strains, can be reliably used for 'typing'.
- the real time RT-PCR assays developed as described in Chapter 4, give reliable detection and identification of the different AHSV serotypes.
- using these methods, five different serotypes of AHSV, namely AHSV-2, 4, 6, 8 and 9, were shown to be involved during the 2010 AHS outbreak in Ethiopia, suggesting that this is an important circulation area for the virus.
- two new serotypes of AHSV (serotypes 6 and 8) were detected in central Africa for the first time.
- Seg-2 nucleotide sequences of AHSV 4, 6 and 8 isolates group in accordance to their geographical origin.
- That no evidence was detected for major topotypes of AHSV (as previously reported for BTV and EHDV).
- AHSV strains causing recent outbreaks in Central and southern Africa have Seg-2 sequences that are distinct from those of the AHSV reference strains and the vaccine strains derived from them.

Chapter 7: Serological reactions between AHSV-9/VP2 and other AHSV serotypes

7.1: Introduction

Modified vaccinia Ankara (MVA) has been widely used as an antigen delivery system providing a number of advantages, including capacity to accommodate extensive amounts of exogenous DNA and the ability to induce both humoral and cellular immunity in the mammalian host (Brun *et al.*, 2008; Merchlinsky & Moss, 1992; Sutter & Staib, 2003) (Chapter 1, section 1.4). It has previously been shown that AHSV VP2 expressed from recombinant MVA (rMVA) can induce antigen specific neutralising antibodies in ponies (Chiam *et al.*, 2009) and protection in mice (Castillo-Olivares *et al.*, 2011).

Phylogenetic relatedness between the genome segments encoding VP2, the primary determinant of AHSV serotype (Bremer *et al.*, 1990) of all nine reference strains of the virus, has been investigated in detail (Chapters 3, 5 and 6) (Potgieter *et al.*, 2003). These investigations show that closer phylogenetic relationships exists between AHSV serotypes 1 and 2; 3 and 7; 5 and 8; 6 and 9 respectively.

The study described in this Chapter was designed to generate an rMVA expressing VP2 of AHSV-9, then use antibodies from ponies vaccinated with this recombinant virus to investigate serological relatedness between VP2 of AHSV-9 and the other monotypic AHSV serotypes.

7.2: Results

7.2.1: Generation of recombinant MVA

Foreign genes were incorporated into MVA using the 'vaccinia virus transfer vector', plasmid pSC11 (Figure 2.2) (Chakrabarti *et al.*, 1985). The plasmid is constructed such that the foreign gene is inserted downstream of the vaccinia virus early / late promoter P7.5. The expression cassette of pSC11 which also contains the LacZ gene, controlled by the late vaccinia promoter P11, is flanked by vaccinia virus thymidine kinase (TK) gene. Homologous recombination within the TK gene can occur when CFE cells, previously infected with MVA, are transfected with pSC11 carrying the foreign gene, resulting in the generation of the recombinant MVA (rMVA). Recombinant MVA viruses are LacZ-positive and can be selected upon staining with β-gal as described in Chapter2, section 2.12.2.

As part of this project, I constructed pSC11 transfer plasmids, containing the VP2 ORF of AHSV-2 (RSArrah/02), AHSV-5 (RSArrah/05), AHSV-6 (RSArrah/06), AHSV-8 (RSArrah/08) and AHSV-9 (PAKrrah/09) (Table 7.1) as described in Chapter 2, section 2.9.2. To validate the sequence integrity of these inserts, each of them was sequenced and compared with the ORF consensus sequence of the respective reference strains (see Chapter 3). However, attempts to engineer pSC11 plasmids containing cDNA copies of AHSV-1 (RSArrah/01), AHSV-3 (RSArrah/3) and AHSV-7 (KENrrah/07) VP2 ORF, only resulted in clones with invalid insert sequence (inserts in the incorrect orientation, truncated, or with premature stop codons) (data not included).

For the purpose of the study, the pSC11/VP2/AHSV-9 transfer vector was used to generate recombinant MVA/AHSV-9/VP2 virus as described in Chapter 2, Section 2.12. Due to limitations on the time available for this project, the remaining constructs are

being used by Dr Javier Castillo-Olivares in wider serological and vaccine studies of AHSV that are continuing at the Pirbright Institute .

Table 7.1: List of vaccinia transfer-plasmids generated: including changes detected relative to their respective / homologous reference strains.

Plasmid annotation	Nucleotide position*	Nucleotide substitution**	Amino acid change
pSC11/VP2/AHSV-2	703	AAC → GAC	Aspargine → Aspartic acid
	1066	ACA → GCA	Threonine → Alanine
	1723	TCA → TTA	Serine → Leucine
pSC11/VP2/AHSV-5	777	TCA → TCG	Serine → Serine
pSC11/VP2/AHSV-6	108	AAA → AAG	Lysine → Lysine
	1725	CTC → CTT	Leucine →Leucine
pSC11/VP2/AHSV-8	288	GTT → GTC	Valine → Valine
pSC11/VP2/AHSV-9	3156	GCT → GCC	Alanine→ Alanine

*Nucleotide position of the ORF in relation to respective reference strain

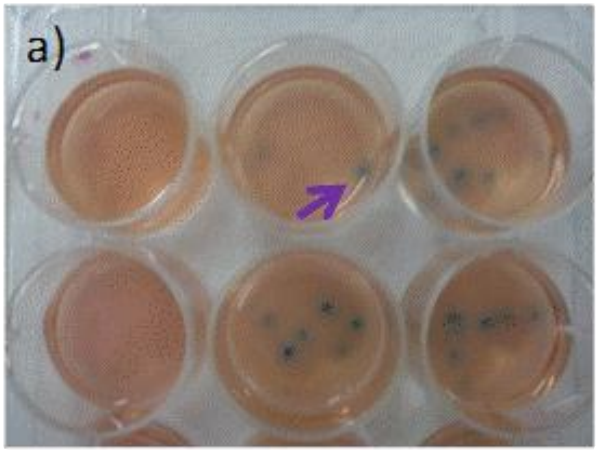
** In relation to respective ORF

7.2.2: Selection of MVA/AHSV-9/VP2

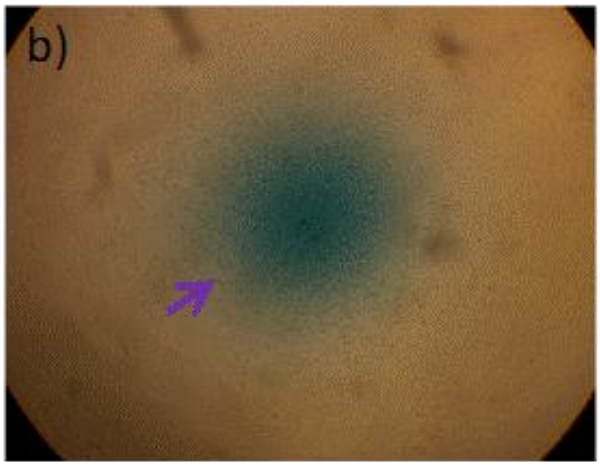
Selection of rMVA containing the AHSV-9 Seg-2 ORF was performed in CEF or BHK TK$^-$ cells, as described in Chapter 2, Section 1.12.2. Initially, cells were infected with the product of transfection / co-infection (see Chapter 2 section 1.12.1) and blue plaque

picks, indicating rMVAs, were collected. Selection of individual, isolated blue plaques was repeated until no more CPE due to wild type vaccinia virus (white plaques) were observed. Representative illustrations of the rMVA selection are shown in Figure 7.1a and 7.1b.

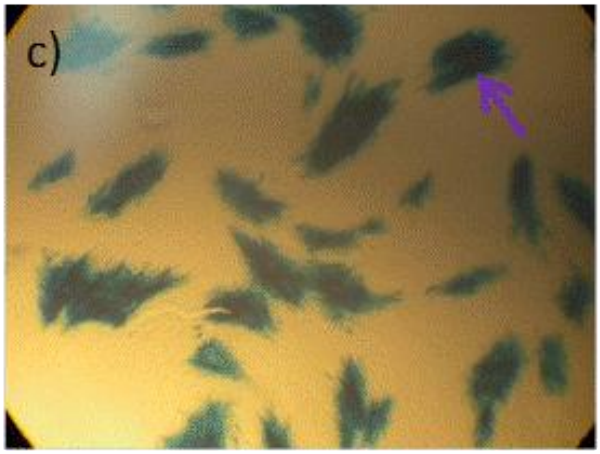
The final titre of MVA/AHSV-9/VP2 was determined by β-Gal Staining (Invitrogen) assay as described in Chapter 2 section 2.12.4 (Figure 7.1 c).



a) Selection of stained (β-Galactosidase positive) individual rMVA plaques. Well defined isolated plaques were collected for further selection.



b) Microscopic examination of rMVA plaque in CEF cells after staining with X-Gal (Invitrogen). No wild type vaccinia plaques are visible.



c) rMVA plaques dyed with β-Gal Staining Kit (Invitrogen) in CEF cells. Plaques were counted to determine the titre final titre of MVA/AHSV-9/VP2.

Figure 7.1: Selection of MVA/AHSV-9/VP2

7.2.3: Functionality of the AHSV-9 VP2 expression cassette in MVA/AHSV-9/VP2.

As specific AHSV-9 VP2 antibodies were not available, the functionality of the MVA/AHSV-9/VP2 expression cassette was confirmed by RT-PCR amplification detection of AHSV-9 VP2 mRNA transcribed by the recombinant virus.

mRNA of MVA/AHSV-9/VP2 was extracted from infected CEF cells (at 24h p.i.) and the RT-PCR reactions were carried out as described in Chapter 2 section 2.12.5. RT-PCR assays using primers specific to AHSV-9 Seg-2 (9A2) (Maan *et al.*, 2011a) gave positive results producing a band of the expected size (1706 bp). The absence of genomic DNA of MVA/AHSV-9/VP2 in the mRNA extracts was confirmed by negative PCR reactions (no RT-step), using the same primer pairs and performed in parallel. The validity of the PCR reaction was 'controlled' using DNA template from PSC11/VP2/AHSV-9, which also resulted in amplification of a band of the correct size (1706 bp) (positive control) (Figure 7.2).

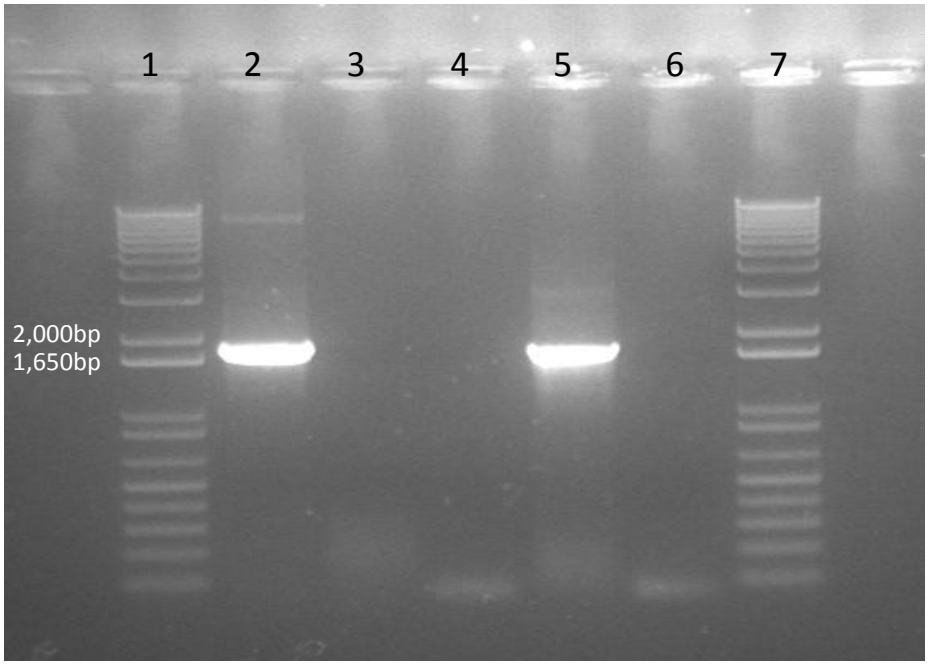


Figure 7.2: Detection of mRNA translated by MVA/AHSV-9/VP2.

1% AGE analysis of PCR and RT-PCR assays confirming the presence and functionality of the MVA/AHSV-9/VP2 expression cassette. Lines 1 and 7 represent TrackIt 1kb Plus Marker (Invitrogen). Lines 1-4 PCR products: 2- pSC11/AHSV-9/VP2, DNA template; 3- MVA/AHSV-9/VP2, mRNA template; 4- PCR no template control. Lines 5 and 6 RT-PCR products: 5- MVA/AHSV-9/VP2, mRNA teplate; 6- RT-PCR no template control. Molecular markers are shown in bp. Expected product size 1706 bp.

7.2.4: Immunisation of ponies

Two Welsh mountain ponies (#464 and #639) received two doses of MVA/AHSV-9/VP2 containing $3x10^8$ pfu per animal, two weeks apart. The rMVA was delivered by a combination of intramuscular and intradermal routes (see Chapter 2 Section 2.13). Local inflammation of the injection sites was observed following the initial administration of rMVA, which resolved within 72h. Both of animals (#464 and #639) displayed slightly elevated rectal temperature (39 and 39.1 ^oC, respectively) which subsided within 24h. These were not recorded after the second immunisation. Blood samples were collected on day 0 of the experiment and at 45 days post immunisation (Figure 7.2) and separated serum was used for subsequent serological investigations.

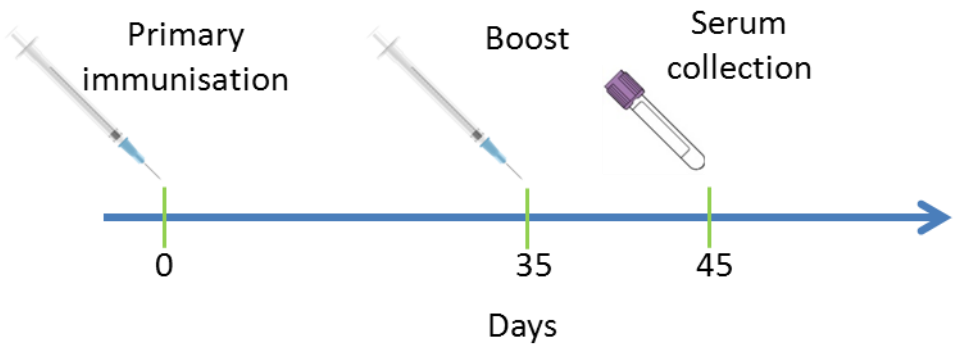


Figure 7.2: Schedule of ponies' immunisation.

7.2.5: Serological relationships between the nine AHSV monotypic reference strains and antisera raised against AHSV-9/VP2

Preliminary tests using antisera raised in ponies against MVA/AHSV-9/VP2 were performed to assess virus neutralising properties of the MVA/AHSV-9/VP2 specific sera. The antisera were tested against each of the nine monotypic AHSV reference strains as described in Chapter 2, Section 2.14. Preliminary screening, using the MVA/AHSV-9/VP2 antisera, diluted 1 in 2, showed that both the homologous AHSV-9 (PAKrah9/03) and a heterologous AHSV-6 (RSArah6/03) strains were neutralised. None of the remaining AHSV viruses was neutralised by these antisera (Table 7.2).

A second serum neutralisation test was conducted using doubling dilutions of the antisera to determine their neutralisation-titre against both AHSV-6 and AHSV-9. This test showed that antisera collected from the ponies on day 0 p.i. did not neutralise AHSV-6 or AHSV-9. However, the antisera collected on day 45 p.i. from ponies #464 and #639 exhibited neutralisation titres of 13 (in both ponies) against AHSV-6, as well as titres of 178 and 106 against AHSV-9.

Table 7.2: Results of preliminary screening AHSV-9 VP2 antisera.

AHSV serotype	AHSV strain	Pony #464	Pony #639
AHSV-1	RSArah1/03	-	-
AHSV-2	RSArah2/03	-	-
AHSV-3	RSArah3/03	-	-
AHSV-4	SPArah4/03	-	-
AHSV-5	RSArah5/03	-	-
AHSV-6	RSArah6/03	+	+
AHSV-7	KENrah7/03	-	-
AHSV-8	RSArah8/03	-	-
AHSV-9	PAKrah9/03	+	+

Sera raised using MVA/AHSV-9/VP2 was tested at a dilution of 1 in 2, with the panel of the nine monotypic reference strains, each representing a different AHSV serotype, as described in Chapter 2 section 2.14. (-) neutralisation not observed; (+) neutralisation observed.

7.3: Discussion

Previous reports have suggested that cross-neutralisation can occur between certain serotypes of AHSV: between serotype 1 and 2; 3 and 7; 5 and 8; 6 and 9 (Coetzer & Guthrie, 2004). Phylogenetic analysis of Seg-2/ VP2 show that each serotype of AHSV groups distinctly from each other such that clear distinctions between serotypes can be made (Chapter 3, section 3.2.3.2). However, the pairs of serotypes that have been reported to cross-neutralise, were also shown to be more closely related to each other than to the remaining serotypes (Chapter 3)(Potgieter *et al.*, 2003).

This Chapter explores the hypothesis that the phylogenetic relationships between Seg-2/VP2 of certain AHSV serotype pairs (1 and 2; 3 and 7; 5 and 8; 6 and 9) as described in Chapter 3 is reflected by serological relationship between sera raised exclusively to VP2

(of AHSV-9, expressed by rMVA) and each of the nine monotypic reference strains respectively.

The replication of MVA in mammalian cells is minimal or non-productive, although the expression of early and late genes is maintained (Sutter & Moss, 1992). Foreign genes can be integrated into MVA's genome without the loss of the virus infectivity and can be expressed after the introduction of the virus into a mammalian host (Meyer *et al.*, 1991). The AHSV-4 VP2 gene has already been successfully expressed in ponies using MVA as the delivery system, showing that it can generate neutralising antibodies via this route (Chiam *et al.*, 2009).

During this study, MVA transfer vectors containing the VP2 gene of AHSV-2, 5, 6, 8 and 9 were made successfully (Table 7.1). While cloning of the VP2 gene into pSC11 (the MVA transfer vector) of most of these AHSV serotypes was a relatively straight forward process, viable bacterial clones containing VP2 of AHSV-1, 3 and 7 were not generated using the same methods (Chapter 2, section 2.9.2). This could possibly be due to some level of VP2 expression in bacterial cells and a toxic effect. Interestingly, Seg-2/VP2 of AHSV-3 and 7 and AHSV-1 and AHSV-2 are phylogenetically related and the protein of these two serotypes both appear to have similar effect (are difficult to clone) in the DH5-α strain of *E. coli*.

For the purpose of this study rMVA containing AHSV-9-VP2 was produced (MVA/AHSV-9/VP2). The recombinant viruses were selected away from wild type virus by a number of rounds of blue-plaque picking, grown in DF-1 cells, titrated and used to raise antisera to AHSV-9VP2 following inoculation into ponies as described. The remaining transfer vectors (Table 7.1) will be used in a study investigating wider serological relatedness between AHSV serotypes.

Analysis of the antibody response after two rounds of vaccination with MVA/AHSV-9/VP2 indicated that the sera could neutralise the homologous AHSV-9 virus (PAKrah9/03) with neutralisation titres of 178 and 106 in two ponies tested. This indicates that the VP2 expressed by rMVA contained functional neutralising epitopes. The AHSV-9/VP2 specific sera also neutralised AHSV-6 (RSArah6/03) (neutralising titre of 13 in both animals), indicating that AHSV-9-VP2 contains epitopes that neutralise both AHSV-9 and the heterologous AHSV-6 virus. None of the other AHSV serotypes was neutralised by this sera. This confirms that the phylogenetic relatedness in Seg-2 / VP2 of AHSV reflects serological relationships (at least for AHSV-9).

Further studies involving precise characterisation of the level of neutralisation are required and will be performed. Time constraints have made it impossible to include these further data in this manuscript. Further analyses, involving VP2 specific sera generated using rMVAs expressing VP2 of the remaining AHSV serotypes will be used to fully explore the hypothesis in future studies.

An in depth analysis of VP2 sequence data (Chapter 3, section 3.2.3.2) has revealed a pattern of insertions / deletions during multiple aa sequence alignment of AHSV VP2. These regions were shown to be located in hydrophilic sites of the protein and co-located with regions of low identity between the nine serotypes of AHSV. These insertion /deletion patterns were mostly serotype related, however they were also similar between the serotype pairs that show serological similarities and a significant level of cross-neutralisation with heterologous sera (Coetzer & Guthrie, 2004). These findings, combined with the ability of AHSV-9 VP2 sera to neutralise both AHSV-6 and AHSV-9, indicate that the insertion / deletion regions could be a part of neutralising epitopes. Further studies of the neutralising properties of these regions, involving introduction of amino acid changes into the insertion / deletion sites and regions neighbouring to them

(by reverse genetics, or rMVA expression of modified proteins) are required to confirm / reject the hypothesis.

Recent in-vivo cross-protection studies showed that heterologous neutralising antibodies were not detected in sera collected from horses (on day 42 and 56 respectively) after two rounds of vaccination with AHSV-6 (n=6) and AHSV-8 (n=6) monotypic live attenuated vaccines (OBP, South Africa) (von Teichman *et al.*, 2010). In the case of the AHSV-6 live attenuated vaccine, homologous neutralising antibodies were not detected in the sera of four out of six vaccinated animals, while the remaining two animals only showed low levels of neutralising antibodies on day 42.

These results contrast with our initial studies of the neutralising properties of the rMVA/AHSV-9/VP2 specific antisera, which neutralised the homologous AHSV-9 and heterologous AHSV-6 viruses. However, further studies concerning the neutralisation properties of VP2 from the remaining AHSV serotypes, would provide more data concerning differences in the neutralising properties of the intact virus, as compared to the neutralising properties of the outer capsid protein VP2 expressed on its own (by rMVA).

The results of these studies might be helpful in the future design of sub-unit, and possibly cross-reactive vaccines against AHSV.

Chapter 8:
General discussion and future work

8.1: Introduction

African horse sickness virus (AHSV) is an arbovirus, which therefore requires the participation of an arthropod vector (*Culicoides spp*. in which the virus also replicates) for transmission between its susceptible equine hosts. Many arboviruses, almost all of which possess an RNA genome, are able to cause disease in humans and/or livestock.

Over the past two decades several arboviruses, including BTV (family *Reoviridae*), West Nile virus (WNV) (family *Flaviviridae*), Rift Valley fever virus (RVFV) (family *Bunyaviridae*), Chikungunya virus (CHIKV) (family *Togaviridae*), and African swine fever virus (ASFV) (the only DNA arbovirus – family *Asfarviridae*) have emerged to cause large epidemics outside their previously endemic distributions, which are frequently severe and dramatic in their outcomes (Costard *et al.*, 2009; Johnson *et al.*, 2012; Weaver & Reisen, 2010). Only last year (2011), Schmallenberg virus (SBV) (family *Bunyaviridae*) was identified for the first time in Germany, as the cause of congenital malformations and still-births in cattle, sheep and goats (Gibbens, 2012).

Many different factors can influence the translocation and spread of arboviruses into previously disease-free areas. These include:

- expansion of the geographic distribution of vectors, due to progressive changes in global climate (Gould & Higgs, 2009; Purse *et al.*, 2008; Weaver & Reisen, 2010; Wilson & Mellor, 2009; Zell *et al.*, 2008);
- passive transportation of infected vectors, e.g. through trade links or via wind-borne dispersal (Gloster *et al.*, 2007; Waldenstrom *et al.*, 2007);

- transportation / movement of infected host animals (Lubroth, 1988; Malkinson *et al.*, 2002; Rodriguez *et al.*, 1992);
- the use (and transmission) of live vaccine strains (Batten *et al.*, 2008b; Sanchez-Vizcaino, 2004).

Where possible, potential routes of arbovirus entry to disease free areas are regulated and efforts are made to maintain contingency and disease prevention plans (DEFRA, 2008, 2011; EEC, 1990, 1992). However the possibility of arbovirus introduction to a non-endemic area, via other routes, is difficult or impossible to control (e.g. passive transportation of infected vectors).

Specific amino acid changes in the genome of Venezuelan equine encephalitis virus (VEEV) (family *Togaviridae*) can have a dramatic impact on virus virulence and consequently on its emergence (Brault *et al.*, 2002). It has also been shown that reassortment between different orbivirus serotypes / strains can generate progeny strains with new properties, potentially leading to the emergence of these viruses in new geographical areas that were previously pathogen-free (Meiring *et al.*, 2009; Moss *et al.*, 1988; O'Hara *et al.*, 1998).

As the emergence, or re-emergence, of arthropod-borne diseases is largely unpredictable (MacLachlan & Guthrie, 2010; Weaver & Reisen, 2010) a key aspect of our preparedness for future outbreaks, is an ability to detect and identify the virus, and therefore monitor its distribution and occurrence.

The geographic regions affected by AHSV are currently limited to sub-Saharan Africa. However, in recent years several dramatic changes have occurred in the distribution of certain arboviruses, most notably the emergence of BTV across the whole of Europe (Saegerman *et al.*, 2008; Wilson & Mellor, 2009). Although, AHSV is transmitted by the

same *Culicoides* species as BTV, it has not emerged from Africa in the last 20 years. However, during the last decade there have been significant changes in the prevalence of the nine different AHSV serotypes detected in central and southern Africa, including the identification of a major new circulation involving five distinct serotypes in Ethiopia (Chapter 1, Section 1.7.1). There have also been dramatic reports from horse owners in South Africa concerning animals dying from the disease, despite vaccination with the OBP polyvalent live-attenuated vaccine (Steyn, 2012) (http://www.ahsequilink.co.za/). This could indicate that these vaccines, which based on AHSV strains that were circulating between 1930 and 1960s (Howell, 1962; McIntosh, 1958), no longer protect against infection with current strain(s) of the virus. It is also possible that a new and previously undescribed serotype of AHSV has emerged. In this context, two novel serotypes of BTV were recently identified and characterised in Switzerland and Oman respectively (Hofmann *et al.*, 2008; Maan *et al.*, 2011b).

8.2: Creation and use of an AHSV sequence database

A major purpose of this study was to generate a database containing full-length / full-genome sequences for each of the nine reference strains of AHSV derived from the Orbivirus Reference Collection, which is held at the Pirbright Institute (http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/AHSV-isolates.htm) (Chapter 3). The sequences of the different AHSV genome segments were compared, between the nine reference strains, to identify nucleotide and amino acid identity levels, as well as phylogenetic relationships and comparisons of hydrophobicity profiles of the encoded proteins (including the recently described NS4).

Although, sequences were already available for some of the individual genome segments of different AHSV isolates (Manole *et al.*, 2012; Potgieter *et al.*, 2009) this thesis

presents the first comprehensive study of the full-genome sequences of all nine AHSV types.

Similar databases have been developed for other orbiviruses of high economic importance to the livestock industry, including BTV and EHDV (Anthony *et al.*, 2009a, b; Anthony *et al.*, 2009c; Maan *et al.*, 2010b). These have proved to be of a great importance for many branches of research, including diagnostic assay development and epidemiology studies of these viruses (Allison *et al.*, 2012; Hofmann *et al.*, 2008; Maan *et al.*, 2012; Maan *et al.*, 2010a; Maan *et al.*, 2011b; Maan *et al.*, 2008; Maan *et al.*, 2010b; Mertens *et al.*, 2007; Nomikou *et al.*, 2009; Shaw *et al.*, 2007).

The initial AHSV sequence database that was generated has already proved to be a valuable tool for diagnostic and molecular epidemiology studies of the virus, providing a basis for the identification of different strains/serotypes of AHSV that are circulating in Africa.

8.3: Analysis of sequence variation in individual AHSV genome segments

The level of nucleotide and amino acid identity in individual genome segments and proteins of different AHSV strains depends on protein function, supporting the hypothesis that different evolution forces act separately on each of the AHSV genome segments. Segments encoding core proteins (VP1, VP3, VP4, VP6 and VP7 - encoded by Seg-1, 3, 4, 7 and 8 respectively) are all highly conserved with >89.1 % nt and >87.6 % aa identity between the different reference strains.

Three of the AHSV non-structural proteins (NS1, NS2 and NS4 - encoded by Seg-5, 9 and 7) are also highly conserved in both their nt and aa sequences (>96.2% nt and >95.7% aa identity). Despite these high identity levels, many of the virus isolates

analysed group independently of each other. However, identical or very similar genome segments were shared between some of the isolates, indicating that reassortment events have occurred in the evolution of these strains (Table 8.1). This confirms the value of the database as a tool for identification of reassortant strains. It also suggests that if more, full genome sequence data of different AHSV isolates becomes available, additional versions / variants of the different genome segments are likely be identified.

Table 8.1: AHSV reference strains with identical genome segments.

Seg-3	Seg-4	Seg-5	Seg-6	Seg-7	Seg-8	Seg-9
SPArrah/04 and, RSArrah/5	SPArrah/04 and RSArrah/5	SPArrah/04 and RSArrah/5	RSArrah/5 and RSArrah/08	RSArrah/01 and RSArrah/06	RSArrah/01 and RSArrah/06;	RSArrah/01 and RSArrah/06;
RSArrah/5 and RSArrah/08					RSArrah/03 and PAKrrah/09	RSArrah/5 and RSArrah/08

Virus isolates sharing the same version of a genome segment are shown in the same colour font.

In contrast, VP2, VP5 and NS3 of AHSV (encoded by Seg-2, 6 and 10) are more variable proteins, and are all thought to be involved in determination of the virulence characteristics of the virus (Huismans *et al.*, 2004; O'Hara *et al.*, 1998). The variability of VP2 and VP5 is thought to be related to their position on the outer surface of the virus particle. Although it is unclear why NS3 is so variable, it has been suggested that it might relate to the use of different insect vector species by the virus (Riegler, 2002) Confirmation of this hypothesis will require further study.

Despite differences in the variability of VP2 and VP5, between different isolates, their phylogenetic relationship are frequently comparable, indicating either a close structural

interaction (Zhang *et al.*, 2010) or functions that in both cases are serotype related (Martinez-Torrecuadrada *et al.*, 1999; Mertens *et al.*, 1989b). However, identical versions of Seg-6 were detected in the isolates of AHSV-5 and AHSV-8 indicating that VP2 and VP5 can reassort independently from each other and that serotype specificity is determined by VP2, not by VP5 of AHSV. Previous phylogenetic studies with BTV, as well as a very recent study showing that viable reassortants can be generated by 'reverse genetics', in which VP2 and VP5 can be readily exchanged between BTV types, confirm this conclusion (Maan *et al.*, 2009; Shaw *et al.*, 2012).

The viral genome exists as a pool containing different versions of each genome segment / gene, with the 'fittest' version in a given environment, representing the major component of the population (Domingo *et al.*, 2012; Elena, 2012; Potgieter *et al.*, 2009). Each of the sequences generated in this study, represents the consensus for an individual genome segment. Consequently the nucleotide at each position in the sequence will be present in the majority (but not necessarily all) of the virus particles in the strain analysed.

The orbivirus non-structural protein, NS4, that was recently identified in BTV (Belhouchet *et al.*, 2011; Ratinier *et al.*, 2011), was present in all serotypes of AHSV. It is encoded by Seg-7, from an alternative ORF which overlaps with that of VP6. In most of the isolates investigated, NS4 is predicted to be 154 aa in length, although in three isolates (RSArrah/02, RSArrah/05 and PAKrrah/09) an alternative stop codon in Seg-7 reduces the length of the protein to 128, 135 and 113 aa respectively. However, detailed examination of sequencing chromatograms showed the presence of a minor nucleotide peak, indicating that the 'full length' NS4 could also be expressed by a proportion of the virus particles in these isolates. These premature stop codons may have been a result of random nucleotide substitution (genetic drift) that have remained uncorrected by the selective pressures (and bottlenecks) of alternative passages in vector and host species.

NS4 has been shown to be highly variable between different species of Orbivirus (Belhouchet *et al.*, 2011) but the functional consequences of these differences have not yet been investigated. Further studies of the function of the truncated versions of AHSV NS4 using reverse genetics and comparisons to the function of the 'full length' proteins, are required.

8.4: The absence of AHSV topotypes

Analysis of the sequences of BTV and EHDV genome segments encoding VP1, VP3, VP4, VP6/NS4, NS1 and NS2, shows that these viruses group in accordance with the geographical origin of the virus, into major eastern and western topotypes (Anthony *et al.*, 2009a, b; Anthony *et al.*, 2009c; Maan *et al.*, 2010b; Pritchard *et al.*, 1995). At the start of this project it was uncertain if AHSV, which is closely related to BTV and EHDV but is restricted to a single continent, would also display similar topotypes. This study shows that variability of most of the AHSV genome segments is significantly lower than described for BTV and EHDV but is similar to that described within a single topotype of these other orbiviruses. This indicates that BTV and EHDV have been circulating on different continents for sufficient time to accumulate large numbers of distinct point mutations, allowing the different topotypes to evolved in these different and isolated geographic locations. However, as AHSV is confined to a single continent, such diversity between different versions of individual genome segments, has not developed (Chapter 3).

The study of identity levels in AHSV Seg-2, involving multiple sequences of serotypes 2, 4, 6, 7, 8 and 9, using sequence data from geographically divergent isolates, provides the first study into genetic variation within the different types of AHSV. Although relatively high overall levels of nt identity were detected within Seg-2 of individual AHSV

serotypes (e.g. >88% for AHSV-9). Despite the absence of major Seg-2 topotypes, the isolates of types 4, 6 and 8 that were examined, do group (within type) in accordance with their geographical location / origins within Africa. This could help to indicate the source of an outbreak strain during epidemiological investigations. An example of this is provided by phylogenetic analyses of the Seg-2 sequences of AHSV-4 isolates from the 1987 outbreak in Spain, which group with AHSV-4 field strains from South Africa (>99% nt identity). This provides evidence for a recent common ancestry of these isolates. This finding is in agreement with epidemiological data that link the outbreak to importation of an AHSV-4 infected zebra from Namibia (a country neighbouring to South Africa) into a Safari Park near Madrid in 1987 (Lubroth, 1988; Rodriguez *et al.*, 1992; Sanchez-Vizcaino, 2004).

8.5: Molecular epidemiology of AHSV

Sequence data for the nine AHSV reference strains (described in Chapter 3), other field and vaccine strains available from the ORC, and publically available Seg-2 sequence data provided an initial sequence database as a basis for molecular typing and further characterisation of novel AHSV isolates (Chapter 6). Novel isolates from the 2010 outbreaks of AHS in Ethiopia were initially identified as serotypes 2, 4, 6, 8 and 9, using real-time RT-PCR assays (Chapter 4) (Aklilu *et al.*, 2012). Subsequent sequencing and molecular epidemiology studies using the database confirmed the serotypes of these strains, as well as providing information concerning movements of different AHSV strains within Africa (Chapter 6).

Most AHSV outbreaks were previously believed to result from the circulation of a single serotype (Coetzer & Guthrie, 2004). The discovery of five different serotypes of AHSV co-circulating in Ethiopia represents a significant addition to our current understanding of

AHSV epidemiology. Previously, AHSV outbreaks involving multiple different serotypes of AHSV had only been reported in South Africa (ProMED-mail, 2009) where all of the serotypes of AHSV are known to circulate (Coetzer & Guthrie, 2004; von Teichman *et al.*, 2010).

Two serotypes of AHSV (serotypes 6 and 8) were also detected for the first time in central Africa, which could reflect either the emergence of these types in previously unaffected areas, or the outcome of better international collaboration on surveillance and diagnosis using the improved diagnostic methods described here. The multiple serotype outbreaks of AHSV identified East Africa as a major circulation area of AHSV, and a potential source of the virus to the Arabian Peninsula, as well as areas further east or in the Mediterranean region. These examples also demonstrate the value of the AHSV database for different applications.

Intra-serotype grouping of AHSV isolates shows that reference strains group together with the vaccine strains that were derived from them, but more distantly from field strains currently circulating in Africa. It would be interesting to investigate if molecular distances, between the current AHSV field strains and the vaccine strains (OBP, South Africa), could be the reason for the lack of protection against AHSV that has recently been reported after vaccination (Steyn, 2012) (http://www.ahsequilink.co.za/).

8.6: Evolutionary selective pressures on the AHSV genes

Sequencing and phylogenetic studies have provided a better understanding of genetic variability within the AHSV species as a whole, showing how evolutionary selective pressures relate to the genetic function of each of the AHSV genome segments / proteins.

All of the AHSV genome segments are under strong purifying selection, the level of which reflects individual gene functions. Thus, VP3 and VP7, the scaffolding sub-core and core-surface proteins of the virus particle, are under the strictest and similar levels of 'purifying selection-pressure' (d_N/d_S of 0.017 and 0.012 respectively) with very low nucleotide substitution rates (Table 3.22). In contrast, NS4, which was non-essential for virus replication in mammalian and insect cells (Ratinier *et al.*, 2011), is under least purifying selection-pressure, with a d_N/d_S value of just 0.883. This confirms that the different AHSV genome segments evolve separately from each other (as also shown for BTV) as expected from a segmented genome virus that can reassort its genome segments (Carpi *et al.*, 2010; Maan *et al.*, 2011b).

In comparison to other AHSV proteins, VP2 is under a relatively lower purifying selection pressure (with d_N/d_S of 0.317), possibly allowing for its adaptation in response to the immune response by the host. The smaller AHSV outer-capsid protein, VP5, appear to be under greater purifying selection than VP2, with a d_N/d_S ratio of 0.067 (compared to 0.317 for VP2). Together with higher sequence identity for VP5, this indicates that the two outer capsid proteins are under different adaptive pressure.

8.7: Serological relationships between AHSV types

Based on previous studies with BTV (Maan *et al.*, 2007a), one of the original hypotheses of this project was that the level of sequence identity in Seg-2/VP2 of AHSV, would reflect serological relatedness between different strains and serotypes. This was confirmed using AHSV-9/VP2 specific sera, to neutralise a panel of monotypic reference strains representing the nine AHSV serotypes.

VP2 is the primary determinant of AHSV serotype (Bremer *et al.*, 1990; Huismans & Erasmus, 1981), and is also the most variable virus protein (55.7% nt and 49.6% aa identities). Phylogenetic comparisons of nt and aa sequences representing each of the known AHSV serotypes, showed that they group separately from each other, with clear separation between serotypes. Higher levels of identity were detected in Seg-2 between AHSV serotypes 1 and 2; 3 and 7; 5 and 8; 6 and 9, with lower levels of relatedness to the remaining isolates (Figures 3.10 and 3.11) (Potgieter *et al.*, 2003). These pairs of serotypes also show low level relationships in cross-neutralisation assays (Coetzer & Guthrie, 2004). This confirms previous studies with BTV, which showed that distinct virus types within the same Seg-2 'nucleotype', were also serologically related (Maan *et al.*, 2009), suggesting the existence of similar nucleotypes for AHSV.

Multiple aa sequence alignments identified insertion / deletion in highly variable regions of the VP2 proteins. Although these appear to be serotype specific, they also showed some similarities between the serotypes that were phylogenetically related. The insertion / deletion regions, in most cases co-locate with predicted hydrophobic regions of the protein, indicating that they might the exposed to the outer surface of VP2. Most of these regions are also located within antigenic regions that have previously been identified for some of the virus serotypes (Bentley *et al.*, 2000; Martinez-Torrecuadrada & Casal, 1995; Martinez-Torrecuadrada *et al.*, 2001; Venter *et al.*, 2000b). However two of these sites (between 190-195 and 765-785 aa) may represent additional antigenic sites (Figure 3.48).

AHSV-5 and AHSV-8 show a significant level of cross-neutralisation (Coetzer & Guthrie, 2004; von Teichman *et al.*, 2010), have an identical Seg-6/VP5, and are phylogenetically more closely related to each other in VP2, than to other AHSV serotypes. It is possible that similarities in the sequence / structural configuration of VP2

from AHSV-5 and AHSV-8 can more readily accommodate a similar VP5 during reassortment and subsequent replication.

Chapter 7 explores serological relatedness between AHSV-9 and other AHSV types using rMVA to express AHSV-9-VP2. Sera raised against the expressed AHSV-9-VP2, neutralised both AHSV-9 and AHSV-6, which are phylogenetically related. Both of these viruses also show similar insertion / deletion patterns in a multiple amino acid sequence-alignment of VP2. This finding supports the hypothesis that the VP2 insertion / deletion patterns could in part reflect the neutralising epitopes of AHSV.

8.8: Diagnostic RT-PCR

High throughput, molecular diagnostic tests (real-time RT-PCR assays) were developed for AHSV detection, as well as discrimination between the different serotypes. The sequence data generated for AHSV (chapters 3 and 6), as well as data already available for Seg-2 of different strains, provided a basis for the development of group-specific real-time RT-PCR assays targeting Seg-1 and Seg-3, as well as type-specific assays targeting Seg-2 (Chapter 4). The ability to rapidly and reliably detect AHSV and identify the virus type causing specific disease outbreaks, is of major importance for the design and implementation of effective control strategies, particularly the selection or development of an appropriate vaccine of the correct serotype.

The selection of a target ‘footprint’ for the group specific RT-PCR assays was guided by analysis of sequence data and the variability between the nine reference strains of AHSV as well as the selection pressure / function of the different proteins. Thus, the group specific assays were designed to detect the most conserved regions of Seg-1 and Seg-3. These assays have been evaluated, optimised and quantified using the admittedly limited range of AHSV isolates available from the ORC.

The diagnostic specificity of these assays have been shown by their ability to detect a wide range of AHSV isolates, representing each serotype of the virus (including field, reference and vaccine strains), from different geographical locations and collected over ~ 50 years. The assays are specific to AHSV and failed to amplify even closely related orbiviruses (EEV, PHSV, EHDV and BTV). Both, Seg-1 and Seg-3 assays are highly 'efficient' and can be applied as quantitative systems. As little as 60 copies of dsRNA, equivalent to 0.029 TCID50 of virus isolate RSArah1/03, were detected in the Seg-1 and Seg-3 assays, with 100% analytical sensitivity. Despite similar physical properties of these AHSV detection systems, the Seg-1 assay was marginally more sensitive.

Real-time RT-PCR assay are highly sensitive but also highly specific tools for detection of the target template. However small number of mismatches between the target sequence and the assays oligos may lead to false negative results (Jiménez-Clavero *et al.*, 2006; Shaw *et al.*, 2007). Therefore the use of these two assays in tandem reduces the possibility of false negative diagnosis. High levels of confidence in true negative results are especially important for screening animals before movement from AHSV endemic areas into AHSV free areas.

At the beginning of the project, no real-time RT-PCR methods were available for the detection of AHSV RNA. Since then a number of different systems targeting different genome segments have been published (Chapter 1, section 1.8.1.3). However mismatches between the target regions and the oligonucleotide components of some of the assays have been shown (Quan *et al.*, 2010). Development of new AHSV detection systems which are made available to researchers and diagnosticians will increase confidence in diagnostic and detection capabilities. Both of these AHSV detection assays have already been applied successfully in research projects, detecting AHSV load in infected mice,

Culicoides midges and tissue culture systems (Castillo-Olivares *et al.*, 2011; Veronesi, 2012).

Chapter 4 also describes the development, optimisation and validation of nine independent real-time RT-PCR assays for the detection of each of the known serotypes of AHSV. This is the first complete set of time real-time RT-PCR assays for AHSV typing, and therefore the first high throughput system, for the characterisation of the nine AHSV types. All the nine assays were optimised to maximise their sensitivity and validated with different isolates from the ORC at the Pirbright Institute. As some of the AHSV serotypes (especially serotypes 1, 3 and 5) were represented by relatively small number of isolates, the diagnostic specificity of the type-specific assays refers mainly to their inability to detect non-homologous serotypes. Each of the respective type-specific assays is also 'efficient' and can be used in dsRNA copy quantification, if required.

These assays are now being applied as front line tools for the identification and typing of AHSV by the Community Reference Laboratory at the Pirbright Institute and were used in the study of a multi-serotype outbreak of AHSV in Ethiopia (Aklilu *et al.*, 2012).

8.9: Monotypic reference stains

During the development and validation of type specific real-time RT-PCR assays, cross-contamination was detected in four of the original AHSV reference strains. In order to facilitate further research, including diagnostic studies and to obtain meaningful results for studies of serological cross-reactions, a new set of plaque-cloned monotypic AHSV reference strains was generated. These were subsequently characterised by sequencing of Seg-2 and Seg-6, encoding the outer capsid proteins VP2 and VP5. These strains have

now been stored in ORC, as RSArah1/03, RSArah2/03, RSArah3/03, SPArah4/03, RSArah5/03, RSArah6/03, KENrah7/03, RSArah8/03 and PAKrah9/03. These new reference strains are accessible from the ORC, Pirbright Institute, UK (Chapter 5) for research and diagnostic work.

8.10: Future work

The investigations described in this thesis provide an initial sequence database that will facilitate molecular characterisation of novel AHSV isolates.

However, only a single strain for AHSV serotypes 1, 3 and 5 are currently available at ORC, the Pirbright Institute. This has made it impossible to assess the level of intra-serotype variation in Seg-2 of these types and thoroughly validate 'typing' real-time RT-PCR assays for detection of these serotypes. The addition of sequence data for other well documented isolates of the different serotypes of AHSV, from different geographical locations, would significantly enhance the sequence database, and help to improve our understanding of the genetic variability of the virus. These additional data is likely to be generated as part of further molecular epidemiology studies of AHSV.

Further validation of the RT-PCR assays developed during this project using additional field strains could potentially identify strains that are not amplified/detected by one or more of the group or type specific assays. The relevant genome segments (or whole genomes) of the isolates would then be sequenced providing a basis for the redesign of the assays to maintain their diagnostic specificity and sensitivity. However, the combined use of two distinct group-specific reactions (targeting Seg-1 and Seg-3) is likely to detect all 'normal' AHSV strains.

The relationship of AHSV-9 to other serotypes was investigated using a recombinant MVA expressing type 9 VP2. Further studies of the serological relatedness of different AHSV strains, using MVA expressing native or modified VP2 of AHSV types 1 to 9 could provide data concerning the identity of putative neutralisation epitopes and inform the development of cross-reactive vaccines that could be safely employed in the control of the virus in non-endemic areas.

Studies involving successive modifications of the insertion / deletion sites that were identified in VP2 by reverse genetics, followed by analyses of the resulting serological / neutralisation properties of the modified protein / virus, would provide a better understanding of the immunological properties of the virus.

The relative significance of functional and serological relationships between Seg-2/VP2 and Seg-6/VP5 of different AHSV serotypes could be further explored using reverse genetic techniques to create reassortants and chimeras between viruses that are less phylogenetically related in Seg-2 and Seg-6 than AHSV-5 and 8. These studies may also contribute to our understanding of the molecular basis for virus neutralisation and serotype.

This thesis makes a significant contribution to research, diagnostic methods, and molecular epidemiology studies of AHSV. It is satisfying that the new techniques and tools developed during the project are already being used by the Non-Vesicular Reference Laboratory at the Pirbright Institute and may therefore contribute to prevention and control of the spread of this economically important, transboundary disease.

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Appendix I

The species of genus *Orbivirus*

Virus species	Number of serotype/strain	Vector species	Hosts species
African horse sickness virus (AHSV)	9 numbered serotypes (AHSV-1 to AHSV-9)	Equids, dogs, elephants, camels, cattle, sheep goats, predatory carnivores and (in special circumstances) humans	*Culicoides* spp.
Bluetongue virus (BTV)	24 numbered serotypes (BTV-1 to BTV-24)	All ruminants, camelids and predatory carnivores (by eating infected meat)	*Culicoides* spp.
Changuinola virus (CGLV)	12 named serotypes	humans, rodents, sloths	Phlebotomines sand flies, culicine mosquitoes
Chenuda virus (CNUV)	7 named serotypes	Seabirds	Ticks
Chobar Gorge virus (CGV)	2 named serotypes	Bats	Ticks
Corriparta virus (CORV)	6 named serotypes/strains[a]	Humans, rodents	Culicine mosquitoes
Epizootic hemorrhagic disease virus (EHDV)	7 numbered serotypes/strains[a] (EHDV-1 and EHDV-3 to EHDV-8, EHDV-1 and EHDV-3 can be grouped together as one 'type')	Cattle, sheep, deer, camels, llamas, wild ruminants, marsupials	*Culicoides* spp.
Equine encephalosis virus (EEV)	7 named or numbered serotypes (EEV-1 to EEV-7)	Equids	*Culicoides* spp.
Eubenangee virus (EUBV)	(4 named serotypes)	Unknown hosts	*Culicoides* spp., anopheline and Culicine mosquitoes
Ieri virus (IERIV)	3 named serotypes	Birds	Mosquitoes
Great Island virus (GIV)	36 named serotypes	Seabirds, rodents, humans	*Argas, Ornithodoros, Ixodes* ticks
Lebombo virus (LEBV)	1 serotype (LEBV-1)	Humans, rodents	Culicine mosquitoes

Orungo virus (ORUV)	4 numbered serotypes (ORUV-1 to ORUV-4)	Humans, camels, cattle, goats, sheep, monkeys	Culicine mosquitoes
Palyam virus (PALV)	13 named serotypes/strains[a]	Cattle, sheep	*Culicoides* spp., Culicine mosquitoes
Peruvian Horse sickeness (PHSV)	1 numbered serotype (PHSV-1)	Horses	Mosquitoes
St'Croix River virus (SCRV)	1 numbered serotype (SCRV-1)	Hosts unknown	Ticks
Umatilla virus (UMAV)	4 named serotypes	Birds	Culicine mosquitoes
Wad Medani virus (WMV)	2 named serotypes	Domesticated animals	*Boophilus, Rhipicephalus, Hyalomma, Argas* ticks
Wallal virus (WALV)	3 serotypes/strains	Marsupials	*Culicoides* spp.,
Warrego virus (WARV)	3 serotypes/strains	Marsupials	*Culicoides* spp., anopheline and culicine mosquitoes
Wongorr virus (WGRV)	8 serotypes/strains	Cattle, macropods	*Culicoides* spp. and mosquitoes
Yunnan Orbiviruses (YUOV)	2 serotypes	Cattle, equids	Mosquitoes
Tentative species			
Andasibe virus		Unknown hosts	Mosquitoes
Codajas virus		Rodents	Mosquitoes
Ife virus		Rodents, birds, ruminants	Mosquitoes
Itupiranga virus		Unknown hosts	Mosquitoes
Japanaut virus		Unknown hosts	Mosquitoes
Kammavanpettai virus		Birds	Unknown vectors
Lake Clarendon virus		Birds	Ticks
Matucare virus		Unknown hosts	Ticks
Tembe virus		Unknown hosts	Mosquitoes
Tracambe virus		Unknown hosts	Mosquitoes

[a] In some species, the serological relationships between strains have not been fully determined. For more information concerning individual named types, see Mertens *et al*. 2005.

Appendix II

Standard solutions

Park Davis Additive Medium (PDAM):
2% (w/v) Bovine serum albumin fraction V (Sigma)
8% (w/v) Calcium lactobionate monohydrate (Sigma)
In GMEM media.

Inoue Transformation Buffer (ITB):
55 mM Manganese chloride,
15 mM calcium chloride,
18.65 g potassium chloride,
20 ml 0.5 M PIPES (pH6.7) (piperazine-1,2-bis[2-ethanesulfonic acid]),
In 1 deionized water .

Luria-Bertani (LB) broth:
1% (w/v) Bacto-tryptone,
0.5% (w/v) 5g Yeast extract,
1% (w/v) NaCl
In deionized water.

Luria-Bertani (LB) agar:
LB broth + 1.5% agar.

SOC medium:
2% w/v Tryptone
0.5% w/v Yeast extract
10 mM KCl
10 mM NaCl
10 mM MgCl2
10 mM MgSO4
20 mM Glucose
In deionized water.

TAE Buffe (50x stock):
242 g Tris Base
57.1 ml Glacial Acetic Acid
100 ml 0.5 M EDTA
In 1 L deionized water

Appendix III

List of sequencing primers used in this study

Y=C+T, R=A+G, W=A+T.

Primer nomenclature	Orientation	5'-Sequence-3'
Genome segment 1		
AHSV/S-1/1-19F	F	GTTTATTTGAGCGATGGTC
AHSV/S-1/302-324F	F	GTTAGATCAATCTTGAARTTAGA
AHSV/S-1/485-507F	F	GAAACAAAGCATAAACCTCTTGG
AHSV/S-1/787-807F	F	GTTGCGYTCACCGTATTCGTG
AHSV/S-1/1051-1070F	F	CCTGAAAATGTTAAAAGCGG
AHSV/S-1/1196-1217F	F	GCGCAGATGAAACCAAGTAGTG
AHSV/S-1/1379-1398F	F	GATGTTTTTAAGCGGTATGG
AHSV/S-1/1705-1725F	F	CGTTGGAGATTTGGAGGCTAC
AHSV/S-1/1918-1941F	F	CGAATTAATAGAGTTTGGATATGG
AHSV/S-1/2137-2157F	F	GGTTTCRCCTACGGATGGAAG
AHSV/S-1/2410-2430F	F	CGAAGCCTCAATGTCAAAAAC
AHSV/S-1/2697-2717F	F	GAGATAGRAAATACGATGATG
AHSV/S-1/2810-2831F	F	GCGATGAATATAATAATGACTG
AHSV/S-1/3038-3059F	F	GCCGTRAGAAGTTTGCCTCTGG
AHSV/S-1/3286-3308F	F	GGCGTTGAAGTTTTTCCCAGATG
AHSV/S-1/3568-3590F	F	GACCGAACGCGTTCGCTTCGATG
AHSV/S-1/3731-3752F	F	GTTGCCCAACATATTATGTTAG
AHSV/S-1/3772-3794F	F	GCCATCACGATATGATATTAATG
AHSV/S-1/3944-3965R	R	GTAAGTGTTTTGAGCTGWGGG
AHSV/S-1/3736-3756R	R	GCATCTAACATAATATGTTGG
AHSV/S-1/3633-3654R	R	GTGCAAACATCCAATGACATTG
AHSV/S-1/3389-3410R	R	GTAGTATTGAGTCAATTCTATC
AHSV/S-1/3172-3192R	R	CTTGGTCTCCAACCTTGCAAC
AHSV/S-1/2811-2831R	R	CAGTCATTATTATATTCATCG
AHSV/S-1/2557-2577R	R	GTTTGAACTTGAGATCTCAGG
AHSV/S-1/2148-2166R	R	GCTAGRTCGCAACCATCCG
AHSV/S-1/1928-1950R	R	CGACCCTCTCCATATCCAAACTC
AHSV/S-1/1705-1725R	R	GTAGCCCTCCAAATCTCCAACG
AHSV/S-1/1502-1521R	R	GTGTTATACTTYAAAGCTAG
AHSV/S-1/1310-1329R	R	GATGTATACATATCTTCAGG
AHSV/S-1/1088-1109R	R	CGTGACTTATTTTTGTTGGATC
AHSV/S-1/781-804R	R	GAATACGGTGARCGCAACGTGTTG
AHSV/S-1/586-606R	R	GAGTAACAATAACCATATTCG
AHSV/S-1/424-444R	R	GGCATATCACCAAAAATCTGC
AHSV/S-1/173-194R	R	CGCGCTCTAAAAACTCCATCATC
Genome segment 2		
AHSV1		
AHSV-1/S-2/1-22F	F	GTTWAWTTCAGCATGGCGTCTG
AHSV-1/S-2/390-409F	F	GCCGGAGAACTATTCAACTG
AHSV-1/S-2/676-696F	F	GCATTGAATGCTCGATATGAG
AHSV-1/S-2/1011-1032F	F	GCAGGCGCTGATTAAGAGTATG
AHSV-1/S-2/1300-1321F	F	GTTGACACAATCATGCTTAGAG
AHSV-1/S-2/1611-1633F	F	GAATATGAAGTCATCTATGAATG
AHSV-1/S-2/2000-2288F	F	GAGATAAAAAGACTGAATATAACG
AHSV-1/S-2/2271-2288F	F	CGCTAGATACCTTAACCG
AHSV-1/S-2/2566-2587F	F	GAGAATATAGATGTTGATTTCG
AHSV-1/S-2/2881-2903F	F	GATTATGTCGTACAACTTCTACC

AHSV-1/S-2/2968-2988F	F	GCAGAGTGGGCTTTACGTGAC
AHSV-1/S-2/3196-3218R	R	GTAWGGGTGATCAGCATGGAACG
AHSV-1/S-2/2880-2903R	R	GGTAGAAGTTGTACGACATAATCG
AHSV-1/S-2/2500-2520R	R	CAATAAACGTGTTGATCCCAC
AHSV-1/S-2/2074-2095R	R	GGTAGATATATGTCGACGCGTG
AHSV-1/S-2/1800-1822R	R	GATCAACGAACAAGTCATCTTCG
AHSV-1/S-2/1499-1519R	R	GTGAAACGGTCTCAACTATTG
AHSV-1/S-2/1097-1118R	R	CGACTGAATCTATCAATATATG
AHSV-1/S-2/667-688R	R	GAGCATTCAATGCGTAATGATG
AHSV-1/S-2/328-348R	R	GATTAATCGCTTGTCATCTAG
AHSV2		
AHSV-2/S-2/1-22F	F	GTTWAWTTCAGCATGGCGTC
AHSV-2/S-2/208-231F	F	GAAACCATGTATTGCCAAATTAAG
AHSV-2/S-2/652-672F	F	GGCCTACTGTATCCACATAGC
AHSV-2/S-2/1003-1024F	F	GTCTTCAGAAATGCCTTAGTTG
AHSV-2/S-2/1543-1565F	F	GGAGATATCATCGACAAATTTGG
AHSV-2/S-2/1860-1881F	F	GGTTGAACGGCATGTGAAATTG
AHSV-2/S-2/2229-2249F	F	GGAGGCGGAGATTTCGCTCAG
AHSV-2/S-2/2569-2591F	F	GAGAACATAGATGTTAATTTTGC
AHSV-2/S-2/3035-3056F	F	GTAAAACTGTCAAGAATGGGAG
AHSV-2/S-2/3221-3199R	R	GTAWGTGTATTCAGCATAGAACG
AHSV-2/S-2/2860-2880R	R	CACTCCACCACAATTCGATCC
AHSV-2/S-2/2400-2424R	R	CACTCCACCACAATTCGATCC
AHSV-2/S-2/2198-2218R	R	CAATCCAACATCTATTGTAGC
AHSV-2/S-2/2189-1911R	R	CTCAACGTTCTCTGTCTGAGC
AHSV-2/S-2/2304-2325R	R	GACTCCATCTAGTATCTTAGCG
AHSV-2/S-2/2018-2038R	R	CTACATCGAACTGCTGGTACC
AHSV-2/S-2/1950-1971R	R	GTGCGTTCTGCGTTCAATTCTG
AHSV-2/S-2/1533-1554R	R	GTCGATGATATCTCCACGGAAG
AHSV-2/S-2/1531-1551R	R	GATATCTCCACGGAAGTTTGG
AHSV-2/S-2/1251-1274R	R	GTTCCATACGGCACATCTGTTCGG
AHSV-2/S-2/1054-1075R	R	CCTCTTTGGCCATCTTCATTTG
AHSV-2/S-2/607-628R	R	GATACAACACATCTTCGTCGTG
AHSV-2/S-2/414-433R	R	GCTCTGCTTGTCTCTTACAG
AHSV3		
AHSV-3/S-2/1-21F	F	GTTWAWTTCACCATGGCTTCG
AHSV-3/S-2/213-235F	F	GATTTATGAACAGATTCGATGCG
AHSV-3/S-2/762-784F	F	CCAAAGGCGGAAGGCTGACGATG
AHSV-3/S-2/1410-1431F	F	GGATGGGGTAAAATGCTACGCG
AHSV-3/S-2/1878-1898F	F	GATAATGATTAAGAAGCGAGG
AHSV-3/S-2/2268-2288F	F	GATCTTCACGAGATATCGAGG
AHSV-3/S-2/2571-2591F	F	GAATGTTGATGTGAGCGCTGC
AHSV-3/S-2/2980-3000F	F	GCAATGAAGAATTTTACAGCG
AHSV-3/S-2/3200-3221R	R	GTAWGTTGATTCACATGGAGCG
AHSV-3/S-2/2883-2905R	R	GTAGAGCTTGGACGACATAATCG
AHSV-3/S-2/2449-2469R	R	GCTCTCTCGCGTCTCATTCCG
AHSV-3/S-2/2023-2046R	R	GAAATCTTCAACATCTATCTCATG
AHSV-3/S-2/1742-1762R	R	CCAAAATTGCTTGATATAACC
AHSV-3/S-2/1267-1288R	R	CTACGGTTCCGTACTCAGCATC
AHSV-3/S-2/846-870R	R	GAATCTTATGACGCTCTCAATAACG
AHSV-3/S-2/307-329R	R	CTATCCACCCTTATTTGGTTGTC
AHSV4		
AHSV-4/S-2/1-18F	F	GTTWAWTTCACCATGGCG
AHSV-4/S-2/340-363F	F	GAAATGAGGAAAATATTGATACAG
AHSV-4/S-2/548-569F	F	GGAGAGATGCGGTTAATGAAAG
AHSV-4/S-2/1007-1029F	F	GACAGGAGATAAGAAAAGCATGG
AHSV-4/S-2/1427-1447F	F	GATGTTATATGTATTGCTTAG
AHSV-4/S-2/1934-1955F	F	GTTTCGTATCATATTGGTATAG

AHSV-4/S-2/2402-2423F	F	GTGTCTTTAACGATGAGAGACG
AHSV-4/S-2/2710-2730F	F	GTCGAACGGATGTGCGCGGAG
AHSV-4/S-2/2991-3013F	F	GCTATCACAGTGGCTGCAAATGG
AHSV-4/S-2/3210-3229R	R	GTAWGTGTATTCACATGGAG
AHSV-4/S-2/2991-3012R	R	CCATTTGCAGCCACTGTGATAGC
AHSV-4/S-2/2667-2687R	R	GATATCGAAAGCAGTGAGTTG
AHSV-4/S-2/2401-2422R	R	GTCTCTCATCGTTAAGACACG
AHSV-4/S-2/2004-2022R	R	CGGCTTCTGTTTCTCATTC
AHSV-4/S-2/1703-1723R	R	CTCCGTAATCTTCCATCACCG
AHSV-4/S-2/1512-1532R	R	GCGACAGTTTCAATGATAGCG
AHSV-4/S-2/1218-1239R	R	GCATGGTTGTCCTCCATTTTCG
AHSV-4/S-2/829-851R	R	GTCTCTCGAGATAAATCATCCCG
AHSV-4/S-2/548-570R	R	CCTTTCATTAACCGCATCTCTCC
AHSV-4/S-2/357-378R	R	CTCACCTGCATACGGCTGTATC
AHSV5		
AHSV-5/S-2/1-22F	F	GTTWAWTTCATCATGGCTTCAG
AHSV-5/S-2/357-379F	F	GATACAACCATACTTCGGTGAGG
AHSV-5/S-2/669-691F	F	GCATTACTATATGCTCAATCCAG
AHSV-5/S-2/1025-1047F	F	GGATACAAGGTATGCCAAAGATG
AHSV-5/S-2/1266-1288F	F	GACGAATAAGAAGTACGGATCCG
AHSV-5/S-2/1516-1536F	F	GTAGAAACAGTGTCCCAAATG
AHSV-5/S-2/1779-1800F	F	GAGCGATATAGCTGATGATACG
AHSV-5/S-2/2016-2016F	F	GTATACACAGTTTGATCCTG
AHSV-5/S-2/2192-2211F	F	GATCGTTAGATTCTTGTTGG
AHSV-5/S-2/2411-2432F	F	GATTAGAGGTAATGGACTATTG
AHSV-5/S-2/2604-2626F	F	GCTGTTGTTATCAACTGAGAAGG
AHSV-5/S-2/2889-2909F	F	GTTACATTTGATTCCTTCACG
AHSV-5/S-2/3099-3122F	F	GGATAGGTTAATACTAATATCTGG
AHSV-5/S-2/3198-3217R	R	GTAWGTGTATTCATCTGGAG
AHSV-5/S-2/2999-2021R	R	GATGAATCCTAAACTACCCTCCG
AHSV-5/S-2/2789-2810R	R	GCACGTCTCTCAACTTCATAGG
AHSV-5/S-2/2583-2605R	R	GCGGCATCCGCTTGTTCGAGTAG
AHSV-5/S-2/2275-2296R	R	GTCGTGCATCATGCTGATATCG
AHSV-5/S-2/1958-1978R	R	GATTCCTTGCTTCAACCTTAG
AHSV-5/S-2/1508-1530R	R	GGACACTGTTTCTACAATTGAAG
AHSV-5/S-2/1260-1281R	R	GTACTTCTTATTCGTCATGATG
AHSV-5/S-2/1030-1050R	R	GTTCATCTTTGGCATACCTTG
AHSV-5/S-2/800-820R	R	GCTCACGCGATGTATTTAGCG
AHSV-5/S-2/596-617R	R	GCATTATGGTCAACGCAATTAG
AHSV-5/S-2/361-380R	R	GCCTCACCGAAGTATGGTTG
AHSV-5/S-2/256-277R	R	GCACGTCGGTAATATAATGTGG
AHSV6		
AHSV-6/S-2/1-20F	F	GTTWAWTTCACCATGGCTTC
AHSV-6/S-2/422-442F	F	GTGAGTCTTTACCAATCTCAG
AHSV-6/S-2/904-924F	F	GATACACTGTCACTCCAGGAG
AHSV-6/S-2/1419-1440F	F	GAGATGTTATGCTTACTGCTTG
AHSV-6/S-2/1865-1880F	F	GATACAGATCAAAATGTGGTAATCG
AHSV-6/S-2/2216-2237F	F	GCCATTGAGCTTTCGATGAGAG
AHSV-6/S-2/2597-2619F	F	GTATCGTCAGAGAAGGGGCTTAG
AHSV-6/S-2/2933-2953F	F	GATGACGTCAATGTACAGTGG
AHSV-5/S-2/3186-3204R	R	GGTWAGTTGAATCACCATG
AHSV-5/S-2/2818-2838R	R	CCACGTGGTCAATCCTTCATAC
AHSV-5/S-2/2399-2360R	R	GATAATAATGCTTATACGTAGG
AHSV-5/S-2/1945-1968R	R	GTTGTTATTTTCTCAACACG
AHSV-5/S-2/1482-1502R	R	GAATTTGATCCCTTCTTGAAG
AHSV-5/S-2/1072-1094R	R	GACCAGTTCGCAATCGGACCTCG
AHSV-5/S-2/698-718R	R	GCGAAGTTCTAGTCACCGTTG
AHSV-5/S-2/310-331R	R	CTCTGTCAACTCGGATCTGATG

AHSV7		
AHSV-7/S-2/-4-16F	F	GGTCGTTTAATTCACTATGG
AHSV-7/S-2/1-22F	F	GTTWAWTTCACTATGGCTTCTG
AHSV-7/S-2/320-341F	F	GAGTAGATAGAGATGATGAAAG
AHSV-7/S-2/376-396F	F	GAAGCGTTCTTCTCGCCGGAG
AHSV-7/S-2/775-795F	F	GGTGCGAAAGGTAATGAATCG
AHSV-7/S-2/844-863F	F	GTGATCGAGAATGTAATACG
AHSV-7/S-2/1160-1179F	F	GGATCGAGCATAAGAAGAAG
AHSV-7/S-2/1510-1530F	F	GTCGAGACAATCTCCCAGATG
AHSV-7/S-2/1762-1783F	F	GGTAAGGAGTGGTCAAATGAGG
AHSV-7/S-2/1980-2002F	F	GATGGCGAGAATCGATATTCATG
AHSV-7/S-2/2274-2294F	F	GAGATATAGGACTGACGATAG
AHSV-7/S-2/2598-2619F	F	GCGTCATCCTTTATTGATATCG
AHSV-7/S-2/2790-2810F	F	GGATCTAAGTTTAGATCGACG
AHSV-7/S-2/2941-2959F	F	GTGTATGGAGAAGATGGAG
AHSV-7/S-2/2969-2989F	F	GGGTTGAGTGGGCGATTAAGG
AHSV-7/S-2/3202-3222R	R	GTAHGGCTGATTCACTTGGAG
AHSV-7/S-2/2896-2918R	R	GGCTTTCGCATTGGCAACTCCTG
AHSV-7/S-2/2714-2735R	R	CTTGCTGCATGATCTGCATGGC
AHSV-7/S-2/2704-2722R	R	CTGCATGGCATATCCTTTC
AHSV-7/S-2/2418-2439R	R	GGTACAAAAACTATACACATCG
AHSV-7/S-2/2071-2091R	R	GATATAAGTTGATGAATGTAG
AHSV-7/S-2/1763-1783R	R	CCTCATTTGACCACTCCTTAC
AHSV-7/S-2/1590-1612R	R	GAACYAACAACTCTTCTGATTCG
AHSV-7/S-2/1164-1184R	R	GCCYTCTTCTTCTTATGCTCG
AHSV-7/S-2/842-862R	R	GTATTACATTCTCGATCACAG
AHSV-7/S-2/439-458R	R	GTTCGCATCTTCGCAACGTG
AHSV-7/S-2/320-341R	R	CTTTCATCATCTCTATCTACTC
AHSV-7/S-2/248-270R	R	GATTACATAATGAGGGAACACCG

AHSV8		
AHSV-8/S-2/1-19F	F	GTTWAWTTCATCATGGCGT
AHSV-8/S-2/437-458F	F	GTGATGTTGAGATGTTGAGGAG
AHSV-8/S-2/887-906F	F	GCACATATACAGGTRCCAAG
AHSV-8/S-2/1407-1427F	F	GCTTGTAGGTGCGTTGAGGTG
AHSV-8/S-2/1845-1867F	F	GGTATTCAGACGTGTTGGACTAG
AHSV-8/S-2/2151-2172F	F	GATCGTGTGGTATGATTTGTCG
AHSV-8/S-2/2495-2514F	F	GAGCATGTGTGGAATCTGAG
AHSV-8/S-2/2839-2860F	F	GAGGGCATAACAACGTGGATTG
AHSV-8/S-2/3198-3220R	R	GTAWGTGTATTCATCATGAGTAG
AHSV-8/S-2/2832-2852R	R	GTTGTTATGCCCTCCATTTTG
AHSV-8/S-2/2510-2530R	R	GTGTTGGGAGAAGGAACTCAG
AHSV-8/S-2/2149-2169R	R	CAAATCATACCACACGATCTC
AHSV-8/S-2/1641-1661R	R	CCGTAATCTCCAACATCAGCC
AHSV-8/S-2/1228-1249R	R	CAACAAGCATTAAATGAGGAAC
AHSV-8/S-2/870-890R	R	GTGCTAAATTTTGAGCCATAG
AHSV-8/S-2/537-557R	R	CTCTCTTTCCATTTGTGTAAC

AHSV9		
AHSV-9/S-2/1-22F	F	GTTWAWTTCACCATGGCGTTCG
AHSV-9/S-2/309-307F	F	CCWCCAAATTAGAGTAGAC
AHSV-9/S-2/591-610F	F	GTGCGTGAGRCATGAACCAG
AHSV-9/S-2/1072-1092F	F	GGTCCTATTCAAAATTGGTCG
AHSV-9/S-2/1538-1558F	F	CGAACTTCAAGCAGGAGATTC
AHSV-9/S-2/1928-1949F	F	GGTTTGTATCATATTGGTATCG
AHSV-9/S-2/2491-2512F	F	GAATCGGAATTCTTGATAGATG
AHSV-9/S-2/2972-2992F	F	GTGATATACTTTCGCTAGAGG
AHSV-9/S-2/3205-3186R	R	GTAWGTGTATTCACATGGAG
AHSV-9/S-2/2794-2814R	R	GCTCATCCTYGGTTCCGCACG
AHSV-9/S-2/2418-2438R	R	GGATCGAGCATCCTAGTACAG
AHSV-9/S-2/1937-1957R	R	GTCGAACTCGATACCAATATG

AHSV-9/S-2/1542-1562R	R	GCTTGAATCTCCTGCTTGAAG
AHSV-9/S-2/1069-1091R	R	GACCAATTTTGAATAGGACCACG
AHSV-9/S-2/587-607R	R	GTTCATGYCTCACGCACATCG
AHSV-9/S-2/312-331R	R	CTTTGTCTACTCTAATTTGG
Genome segment 3		
AHSV/S-3/1-22F	F	GTTWATTTTCTGCTGTGAACTC
AHSV/S-3/408-429F	F	GGATTTTATGCATTGATACTGG
AHSV/S-3/690-712F	F	GTATTACAAGGATATATTGAAGG
AHSV/S-3/947-968F	F	GCCCAACCCAAGAATTAATTCG
AHSV/S-3/1290-1311F	F	GGTCCAACAGGTCAACCCCTAG
AHSV/S-3/1566-1586F	F	GATCAAGAAGCAGCTTATTTG
AHSV/S-3/1851-1871F	F	GCTGAATTATCCACGATGCAG
AHSV/S-3/2238-2259F	F	GGAGCGCCACCAATTGGAGATG
AHSV/S-3/2536-2558F	F	CAAATACGCCAGATACTTTGATC
AHSV/S-3/2770-2792R	R	GTAAGTGTAATTCTGCCCGCTAG
AHSV/S-3/2542-2560R	R	GTGATCAAAGTATCTGGCG
AHSV/S-3/2247-2267R	R	GTGAGTAACATCTCCAATTGG
AHSV/S-3/1864-1884R	R	GCTGTACATCCATCTGCATCG
AHSV/S-3/1422-1444R	R	GAACATGATCATATGGACTCG
AHSV/S-3/1189-1210R	R	GAGTTATAGTTTGTCATGATCG
AHSV/S-3/838-859R	R	GCATTAATCGGTAACCTTTGTG
AHSV/S-3/616-717R	R	GTATACCTTCAATATATCCTTG
AHSV/S-3/417-437R	R	CAATTTCTCCAGTATCAATGC
AHSV/S-3/168-187R	R	GCCATATCGCTCTCTGATTTTG
Genome segment 4		
AHSV/S-4/-6-15F	F	GAGGTCGTTTATTTAGGATGG
AHSV/S-4/1-24F	F	GTTTATTTAGGATGGAACCTTACG
AHSV/S-4/51-70F	F	GAATACCTACTCAAAGATAG
AHSV/S-4/320-344F	F	GTTAATTGGCAGAAGAAGGGTAACG
AHSV/S-4/526-550F	F	GACCAACCGATAAGAAATTAGTTAG
AHSV/S-4/732-752F	F	GTTGTGTCTGCGCGTGATTTG
AHSV/S-4/879-897F	F	GCGGAAGCGTTGTGTGCAG
AHSV/S-4/1173-1192F	F	CATATTGAGCGCGCTGATGG
AHSV/S-4/1325-1347F	F	GCAGAATTTCACATATGATGATG
AHSV/S-4/1679-1698F	F	GTCAAATTCATATGTTGCTG
AHSV/S-4/1695-1717F	F	GCTGATCTTGATTGGTGGTTTCG
AHSV/S-4/1962-1978R	R	GTAAGGTTATTAGGATT
AHSV/S-4/1916-1936R	R	GCGTTACGCAWATATTGTATC
AHSV/S-4/1701-1724R	R	GATCATACGAAACCACCAATCAAG
AHSV/S-4/1515-1536R	R	GAACCGGACTCGTTAACGCATG
AHSV/S-4/1326-1349R	R	GACATCATCATATGTGAAATTCTG
AHSV/S-4/1099-1122R	R	GATACTCRTCAACTAATCTTCTCG
AHSV/S-4/656-674R	R	GCATATCCATTTRATYCTG
AHSV/S-4/578-597R	R	CGACATAATACACTTCATCC
AHSV/S-4/319-343R	R	GTTACCCTTCTTCTGCCAATTAACG
AHSV/S-4/240-261R	R	GTGAAATCGTGCAATCCTTCAG
Genome segment 5		
AHSV/S-5/1-17F	F	GTTAAAGAACCTAGGCGG
AHSV/S-5/300-321F	F	GCGGATTACGTTAGTCAATATG
AHSV/S-5/356-376F	F	GTATGATGTCTATCTCCAGAG
AHSV/S-5/488-505F	F	GAATCCAGCTCCAGTTGC
AHSV/S-5/588-606F	F	GGTTTAGCTCAAGCTATAG
AHSV/S-5/755-773F	F	GGCACAGGATTTCAAATTC
AHSV/S-5/801-819F	F	CCAAGATTGTTGCGACAAC
AHSV/S-5/849-866F	F	GTGATTCATCATGTGATG
AHSV/S-5/971-990F	F	GATGMGGACAGCRAGGTTGG
AHSV/S-5/1073-1095F	F	GCTTGACACAATCCCRGTGGTTG

AHSV/S-5/1329-1353F	F	GATGTTTCACCAATCAATGGCTCTG
AHSV/S-5/1554-1575F	F	GATGATGAGGATGGCGATGTTG
AHSV/S-5/1735-1749+8R		CAGAGGTCGTAAGTTTGTGAAC
AHSV/S-5/1732-1749R	R	GTAAGTTTGTGAACCAGG
AHSV/S-5/1627-1647R		CTGGCCAACCTGTTGTATTTC
AHSV/S-5/1328-1351R	R	GAGCCATTGATTGGTGAAACATCG
AHSV/S-5/1044-1067R	R	GAGGTCAACCATAAAGCATACCTG
AHSV/S-5/873-898R	R	GTATCTTCATCATAATATCRTAAATG
AHSV/S-5/510-532R	R	CCAAATTGACCCCATCGGCTAAG
AHSV/S-5/354-372R	R	GGAGATAGAGACATCATACAG

Genome segment 6		
AHSV1-9		
AHSV1-9/S-6/1-22F	F	GTTWATTTTTCCAGARRCCATG
AHSV1		
AHSV-1/S-6/271-290F	F	GCCAGATCCTTTGAGTCCTG
AHSV-1/S-6/425-445F	F	GGAGAGGAAAAGATCGAAGAG
AHSV-1/S-6/568-589F	F	GACGGAAGACGAGATCGCAATG
AHSV-1/S-6/825-845F	F	GATCTATCGCATCTCAAGGTTG
AHSV-1/S-6/940-961F	F	GGTCGACGAAATGAATCAGGAG
AHSV-1/S-6/1158-1178F	F	GTAGTTTCATGATTGGCTTTG
AHSV-1/S-6/1359-1379F	F	GGTCGCATCCAATATATATGG
AHSV-1/S-6/1543-1565R	R	GTAAGTGTTTTTCCCGCCCACAG
AHSV-1/S-6/1154-1174R	R	GCCAATCATGAAACTACGCTG
AHSV-1/S-6/833-853R	R	GATCTCCGCAACCTTGAGATG
AHSV-1/S-6/511-532R	R	GTTGGTGATGCGTTTTGATTGG
AHSV-1/S-6/230-252R	R	GCCACATTTAAGATTACCGCTTG
AHSV2		
AHSV-2/S-6/127-145F		GAGAGTGGTTGAAAGTGAG
AHSV-2/S-6/673-694F	F	GGAGATGCTTGACATAAGTGCG
AHSV-2/S-6/973-995F	F	GATACAGTCTATATTACCCTTAG
AHSV-2/S-6/1325-1345F	F	GAGTTGCATAAGCGTCGGATG
AHSV-2/S-6/1435-1456F	F	GGTACATAACACTGATCTGCAG
AHSV-2/S-6/1543-1565R		GTAAGTGTTTTTCCCGCCCACAG
AHSV-2/S-6/1493-1515R	R	GAAGCGCGTTCATTATCGCTCCG
AHSV-2/S-6/1215-1235R	R	GCATTATGTGAGCCTCAACTG
AHSV-2/S-6/908-927R	R	GACTTGATTGCAACCGCTAG
AHSV-2/S-6/703-722R	R	CTTCTTCGCTTGCTGTTTCG
AHSV-2/S-6/671-693R	R	GCACTTATGTCAAGCATCTCCTG
AHSV3		
AHSV-3/S-6/326-348F	F	GAAAGAGCGGAGAAGGAAGATCG
AHSV-3/S-6/560-580F	F	GATTTAAGAACTTCGGATGAG
AHSV-3/S-6/799-819F	F	GATAATAGATAAACTCACAGG
AHSV-3/S-6/1054-1075F	F	GAAAATACATTCAGARCATACG
AHSV-3/S-6/1277-1298F	F	GCTTATAGTGAGTTCATGAACG
AHSV-3/S-6/1411-1432F	F	GCTCGTTTCGAACGCGATGAAG
AHSV-3/S-6/1545-1567R	R	GTATGTGTTTTYYCCGCGCCRTG
AHSV-3/S-6/1278-1299R	R	GCGTTCATGAACTCACTATAAG
AHSV-3/S-6/970-990R	R	GGCATGATGGACTWTATAACG
AHSV-3/S-6/747-767R	R	GTGTCGTCGCAACAACATTTG
AHSV-3/S-6/502-521R	R	GCTCAGATTGATCTTTYCCG
AHSV-3/S-6/279-299R	R	GTTGTTCACCTGGGCTTAATG
AHSV4		
AHSV-4/S-6/215-235F	F	GGTGATTCAATTAAGCAGGCG
AHSV-4/S-6/518-540F	F	GAACGCATTACAAAGCTATATCG
AHSV-4/S-6/848-869F	F	GACATTCATCCACACATCATTG

AHSV-4/S-6/1139-1161F	F	GCACCRCATCATCAACAACGAAG
AHSV-4/S-6/1380-1401F	F	GATCGATGGATTACGCTATAAG
AHSV-4/S-6/1547-1567R	R	GTATGTGTTTTCTCCGCGCCG
AHSV-4/S-6/1343-1362R	R	GTTCCCATACTCYTTTGTAG
AHSV-4/S-6/1043-1061R	R	GTATCTTCAATGCACCTGG
AHSV-4/S-6/876-897R	R	GTTACAGTATCACGTAGCATTG
AHSV-4/S-6/521-541R	R	GCGATATAGCTTTGTAATGCG
AHSV-4/S-6/350-370R	R	CGCATTGTGTGTTTCAATCAC
AHSV-4/S-6/215-235R	R	CGCCTGCTTAATTGAATCACC
AHSV5		
AHSV-5/S-6/275-295F	F	GATCCGTTAAGTCCAGGCGAG
AHSV-5/S-6/503-522F	F	GGAAAGGATCAGTCAGAACG
AHSV-5/S-6/746-766F	F	GCAAACGTTGTCGCGACAACG
AHSV-5/S-6/940-959F	F	GATCGATGAAATGAATACGG
AHSV-5/S-6/1141-1161F	F	GCCGCATCATCAACAGAAGAG
AHSV-5/S-6/1277-1298F	F	GCTTATAGTGAGTTTATGAATG
AHSV-5/S-6/1544-1566R		GTATGTGTTTTTCCCGCGCCATG
AHSV-5/S-6/1325-1345R		CAATCTCCTCTTATGTAACTC
AHSV-5/S-6/1120-1142R	R	GCGCAATGCATCTACATATAAAG
AHSV-5/S-6/896-918R	R	GCCATAGCTAACTCATTATCTGG
AHSV-5/S-6/791-812R	R	GCTTATCTATGATCTCCTTAAG
AHSV-5/S-6/671-691R	R	GCTTAGGTCCAACATCTCCTG
AHSV-5/S-6/521-541R	R	GCGATATAGTTGGGTGATACG
AHSV-5/S-6/296-318R	R	GACACCTTATTATACAGAAGCTG
AHSV-5/S-6/133-154R	R	GCTACCAACTTCGCTTTCCACG
AHSV6		
AHSV-6/S-6/168-190F	F	GAGTAATGCAAGGAACAATTCAG
AHSV-6/S-6/411-431F	F	GGAAAATAATGAAGGGAGAGG
AHSV-6/S-6/635-655F	F	GAACTTGAACAGCAGGCAACG
AHSV-6/S-6/916-935F	F	GGCGATAAARTCGAAGGTTG
AHSV-6/S-6/1129-1148F	F	CAGATGCATTGCGCCRCATC
AHSV-6/S-6/1369-1388F	F	GATTTATATGGGATCGATGG
AHSV-6/S-6/1544-1566R	R	GTATGTGTTTTYYCCGCGCCRTG
AHSV-6/S-6/1364-1383R	R	GATCCCATATAAATCGGATG
AHSV-6/S-6/1050-1070R	R	GCTCTGAATGTATTTTCAACG
AHSV-6/S-6/799-819R	R	CCTGTGAGTTTATCTATTATC
AHSV-6/S-6/573-592R	R	GATSATCCTCGTCTCATCCG
AHSV-6/S-6/252-272R	R	GAGCCGACTCTAACGTGCCAG
AHSV7		
AHSV-7/S-6/215-235F	F	GGAGATTCAATCAAACAAGCG
AHSV-7/S-6/503-522F	F	GGTAAAGATCAATCAGATCG
AHSV-7/S-6/704-722F	F	GAAACAGCGGCTGAAGAAG
AHSV-7/S-6/898-917F	F	GGATAGCGATTTAGCGATGG
AHSV-7/S-6/1161-1184F	F	GCTTCATGATTGGTTTCGATCTGG
AHSV-7/S-6/1544-1566R	R	GTAAGTGTTTTTCCCGCCCCATG
AHSV-7/S-6/1219-1230R	R	CCATGCATCACATGACTCTCC
AHSV-7/S-6/1000-1021R	R	CTCGTGCTTATCATACTCCTTC
AHSV-7/S-6/747-767R	R	GAGTTGTTGCCACAACATTCG
AHSV-7/S-6/595-618R	R	GCATTGAACTTCTCTCTATATTCG
AHSV-7/S-6/377-397R	R	CTCCTTCCCATACTTTTCAAC
AHSV8		
AHSV-8/S-6/218-238F	F	GATTCAATCAGACAGGCAGTG
AHSV-8/S-6/350-368F	F	GTGATTGAAACGCACAATG
AHSV-8/S-6/518-540F	F	GTGATTGAAACGCACAATG
AHSV-8/S-6/690-710F	F	GCGCAGAGGTCATTGAGACAG
AHSV-8/S-6/911-932F	F	GCTATGGCGATAAAATCAAAGG
AHSV-8/S-6/1082-1104F	F	GTTCATATATATACGACGCCCTG

AHSV-8/S-6/1325-1345F	F	GAGTTACATAAGAGGAGATTG
AHSV-8/S-6/1380-1401F	F	GATCAATGGATTACACCGTTAG
AHSV-8/S-6/1545-1567R	R	GTATGTGTTTTCTCCGCGCCGTG
AHSV-8/S-6/1512-1534R	R	GCTATTTTCACACCAAATAGAAG
AHSV-8/S-6/1076-1098R	R	GTCGTATATATATGAACTTTCGG
AHSV-8/S-6/943-963R	R	GTTTCCGTATTCATTTCATCG
AHSV-8/S-6/693-714R	R	GCTGCTGTCTCAATGACCTCTG
AHSV-8/S-6/548-570R	R	GTTCTCAGATCCTCCTCTGTCTG
AHSV-8/S-6/296-318R	R	GCCACCTTATTATACAGAAGCTG
AHSV-8/S-6/163-183R	R	GTACCTTGCATCACTCCATCG
AHSV9		
AHSV-9/S-6/170-190F	F	GTAATGCAAGGAACAWTTCAG
AHSV-9/S-6/327-349F	F	GAAAGAGCGGAGAAGGAAGATCG
AHSV-9/S-6/568-587F	F	GAACTTCGGATGARACGAGG
AHSV-9/S-6/800-820F	F	GATAATWGATAAACTCACWGG
AHSV-9/S-6/1036-1056F	F	GTRAATATACCAAGTGCGTTG
AHSV-9/S-6/1288-1308F	F	GARTTCATGAACGCGGCTTGG
AHSV-9/S-6/1546-1568R	R	GTATGTGTTTTCYCCGCGCCRTG
AHSV-9/S-6/1131-1150R	R	GATGYGGYGCAATGCATCTG
AHSV-9/S-6/858-877R	R	GCTTTCTCAATTATRTGAGG
AHSV-9/S-6/627-648R	R	GCTGTTCAAGTTCAATYGCCTG
AHSV-9/S-6/331-351R	R	CACGATCTTCCTTCTCCGCTC
AHSV-9/S-6/156-176R	R	GCATTACTCCATCWATCGCCG
Genome segment 7		
AHSV/S-7/1-20F	F	GTTAAATAAGTTGTCTCATG
AHSV/S-7/198-218F	F	GATCGAAGAGTGGAGAGCCAG
AHSV/S-7/425-444F	F	GACCGGAGCAGATCGTGTCG
AHSV/S-7/643-666F	F	GGATTGGAGGATTAGCAACGCAGG
AHSV/S-7/782-804F	F	GGAAGACATTCTACATCAATCAG
AHSV/S-7/1169-1147R	R	GTAAGTTTTAAGTTGCCCTCAAG
AHSV/S-7/695-718R	R	GAAAACACCTGAACTTCAACTCCG
AHSV/S-7/500-522R	R	GGCCTTGAAGACCTCCTGAAGTG
AHSV/S-7/192-215R	R	GCTCTCCACTCTTCGATCATCATG
Genome segment 8		
AHSV/S-8/-6-13F	F	GAGGTCGTTWAAATTCGG
AHSV/S-8/1-17F	F	GTTWAAATTCGGTTAGG
AHSV/S-8/264-288F		GATTATGATCARGCGTTGGCAACTG
AHSV/S-8/270-290F	F	GATCARGCGTTGGCAACTGTG
AHSV/S-8/357-376F	F	GGTCAAATGCAAACATTCGG
AHSV/S-8/414-433F	F	GTTGAGGTGCAACAATCTGG
AHSV/S-8/654-675F	F	GTCACCGTTGATGGAGTAAATG
AHSV/S-8/681-700F	F	GCTGGAGATGTCGTYGCATG
AHSV/S-8/815-835F	F	GGCTCCAACGCTCACAAGATG
AHSV/S-8/893-914F	F	GAATATGCAGCCWATTAATCCG
AHSV/S-8-2/561-581R	R	CCAAACGAAATAGATCATGAC
AHSV/S-8/1179-1160R	R	GTAAGTGTATTCGGTATTG
AHSV/S-8-2/998-1021R	R	CCATTCATTCTGAAATCTGGTCTC
AHSV/S-8-2/737-758R	R	CTGTAAAATTGATCTGCGTGCC
AHSV/S-8/712-732R	R	GATTTCCAACATTCACTGGTG
AHSV/S-8-2/470-492R	R	CAGCAATATTTGAATTGATGTAC
AHSV/S-8/443-460R	R	GTTCGACCTTGCGGTACG
AHSV/S-8/153-174R	R	GTGGCCTCATCGATACCGAATG
Genome segment 9		
AHSV/S-9/-8-13F	F	CAGAGGTCGTAAGTGTAATTC
AHSV/S-9/17-36F	F	GTCATCATGGCAGAGGTCAG
AHSV/S-9/1-17F	F	GTTTAAAAATCCGTTCG

AHSV/S-9/392-411F	F	GTGAGAGGAAGCGGTGCGGT
AHSV/S-9/540-560F	F	GATGGAACGAGCCCTGAGTGG
AHSV/S-9/873-892F	F	GGTTGAACGAGGTAAAGACG
AHSV/S-9/1142-1166R	R	GTATGTTGAAATCCGCGGTTACATG
AHSV/S-9/1165-1148R	R	GTATGTTGAAATCCGCGG
AHSV/S-9/685-703R	R	GTGCCACATCATCTGAACG
AHSV/S-9/422-439R	R	GATCCATTCCATTCTTCG
AHSV/S-9/217-238R	R	GTCTTGAATACGGTAAGCTCCG

Genome segment 10		
AHSV seg-10 α+β+γ		
AHSV-α+β+γ/S-10/1-22F	F	GTTTAAATTATCCCTTGTCATG
AHSV-α+β+γ/S-10/157-179F	F	GTCGCTTGGGATACTTAAYCAAG
AHSV-α+β+γ/S-10/734-758R	R	GTAAGTCGTTATCCCGGCTCCCTAG
AHSV-α+β+γ/S-10/159-180R	R	GCTTGYTTAAGTATCCCAAGCG
AHSV seg-10 α		
AHSV-α/S-10/622-645F	F	GTATGTCRTGGARTGGAGAYACTG
AHSV-α/S-10/495-517R	R	GTACATAAYAARTTCAGYCCATG
AHSV seg-10 β		
AHSV-β/S-10/504-523F	F	GTACCACTATGTTGTTAGCG
AHSV-β/S-10/456-758R	R	CGACAGCCAACCATCTCCAC
AHSV seg-10 γ		
AHSV-γ/S-10/508-529F	F	GTTATGTACCACGATGCTACTG
AHSV-γ/S-10/516-534R	R	GCTGCCAGTAGCATCGTGG

Appendix IV

List of publication during D.Phil studies.

Publications:

Aklilu, N., Batten C., Gelaye E., Jenberie S., Ayelet G., Wilson. A., Belay A., Asfaw Y., Oura C., Maan S., **Bachanek-Bankowska K.** & Mertens P. P. C. (2012). African horse sickness outbreaks caused by multiple virus types in Ethiopia. *In preparation*.

Oura CA, Ivens PA, **Bachanek-Bankowska** K, Bin-Tarif A, Jallow DB, Sailleau C, Maan S, Mertens PC, Batten CA.(2012) African horse sickness in The Gambia: circulation of a live-attenuated vaccine-derived strain. Epidemiol Infect. 2012 Mar;140(3):462-5.

Maan S, Maan NS, Nomikou K, Veronesi E, **Bachanek-Bankowska** K, Belaganahalli MN, Attoui H, Mertens PP. (2011) Complete genome characterisation of a novel 26th bluetongue virus serotype from Kuwait. PLoS One. 2011;6(10):e26147.

Maan NS, Maan S, Nomikou K, Belaganahalli MN, **Bachanek-Bankowska** K, Mertens PP. (2011) Serotype specific primers and gel-based RT-PCR assays for 'typing' African horse sickness virus: identification of strains from Africa. PLoS One. 2011;6(10):e25686.

Castillo-Olivares J, Calvo-Pinilla E, Casanova I, **Bachanek-Bankowska** K, Chiam R, Maan S, Nieto JM, Ortego J, Mertens PP. (2011) A modified vaccinia Ankara virus (MVA) vaccine expressing African horse sickness virus (AHSV) VP2 protects against AHSV challenge in an IFNAR -/- mouse model. PLoS One. 2011 Jan 26;6(1):e16503.

Darpel KE, Batten CA, Veronesi E, Williamson S, Anderson P, Dennison M, Clifford S, Smith C, Philips L, Bidewell C, **Bachanek-Bankowska K**, Sanders A, Bin-Tarif A, Wilson AJ, Gubbins S, Mertens PP, Oura CA, Mellor PS. (2009) Transplacental transmission of bluetongue virus 8 in cattle, UK. Emerg Infect Dis. 2009 Dec;15(12):2025-8.

Anthony SJ, Maan S, Maan N, Kgosana L, **Bachanek-Bankowska** K, Batten C, Darpel KE, Sutton G, Attoui H, Mertens PP. (2009) Genetic and phylogenetic analysis of the outer-coat proteins VP2 and VP5 of epizootic haemorrhagic disease virus (EHDV): comparison of genetic and serological data to characterise the EHDV serogroup. Virus Res. 2009 Nov;145(2):200-10.

Reid SM, Ebert K, **Bachanek-Bankowska** K, Batten C, Sanders A, Wright C, Shaw AE, Ryan ED, Hutchings GH, Ferris NP, Paton DJ, King DP. (2009) Performance of

real-time reverse transcription polymerase chain reaction for the detection of foot-and-mouth disease virus during field outbreaks in the United Kingdom in 2007. J Vet Diagn Invest. 2009 May;21(3):321-30.

Batten CA, Sanders AJ, **Bachanek-Bankowska** K, Bin-Tarif A, Oura CA.(2009) Bluetongue virus: European Community proficiency test (2009) to evaluate ELISA and RT-PCR detection methods with special reference to pooling of samples. Vet Microbiol. 2009 Mar 30;135(3-4):380-3.

Batten, C.A., **Bachanek-Bankowska, K.**, Bin-Tarif, A. , Kgosana, L. , Swain, A.J. , Corteyn, M. , Darpel, K. , Elliott, H.E. and Oura, C. Bluetongue virus: inter-laboratory comparison tests to evaluate ELISA antibody and RT-PCR detection methods.Vet Microbiol. 2008; 129(1-2):80-8.

Conference presentations:

Oral Presentation: *African horse sickness virus: Novel typing real-time RT-PCR assays show co-circulation of multiple strains in sub-Saharan Africa*. **Veterinary Research Club**, Newmarket, UK. 22nd June 2012.

Oral Presentation: *African horse sickness virus: current molecular-epidemiological situation in sub-Saharan Africa.* **Epizone, 5th Annual Meeting**, Arnhem, The Netherlands. 11-14 April 2012.

Poster: *Sequence analyses and phylogenetic comparisons of the genome segments 2 and 6 of different serotypes of African horse sickness virus.* **Epizone, 4th Annual Meeting**, Saint Malo, France. 7-10 June 2010.

Poster: *Development of a molecular database and reference collection for AHSV.* **MedReoNet**, Lisbon, Portugal. 2-4 December 2009.

Appendix V:
The standard genetic code

The standard genetic code:

TTT phe F	**TCT** ser S	**TAT** tyr Y	**TGT** cys C
TTC phe F	**TCC** ser S	**TAC** tyr Y	**TGC** cys C
TTA leu L	**TCA** ser S	**TAA** STOP **Z**	**TGA** STOP **Z**
TTG leu L	**TCG** ser S	**TAG** STOP **Z**	**TGG** trp W
CTT leu L	**CCT** pro **P**	**CAT** his H	**CGT** arg R
CTC leu L	**CCC** pro **P**	**CAC** his H	**CGC** arg R
CTA leu L	**CCA** pro **P**	**CAA** gln Q	**CGA** arg R
CTG leu L	**CCG** pro **P**	**CAG** gln Q	**CGG** arg R
ATT ile I	**ACT** thr T	**AAT** asn N	**AGT** ser S
ATC ile I	**ACC** thr T	**AAC** asn N	**AGC** ser S
ATA ile I	**ACA** thr T	**AAA** lys K	**AGA** arg R
ATG START M	**ACG** thr T	**AAG** lys K	**AGG** arg R
GTT val V	**GCT** ala A	**GAT** asp D	**GGT** gly G
GTC val V	**GCC** ala A	**GAC** asp D	**GGC** gly G
GTA val V	**GCA** ala A	**GAA** glu E	**GGA** gly G
GTG val V	**GCG** ala A	**GAG** glu E	**GGG** gly G

Uncertainty codes:

R	AG
Y	CT
M	AC
K	GT
S	CG
W	AT
H	ACT
B	CGT
V	ACG
D	AGT
N	ACGT