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Complete genome comparison of the recent and historic field strains of African horse sickness virus isolated over four decades.

by

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Submitted in partial fulfilment of the requirement for the degree
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Declaration

I, Ralengopeng Nick Mokotoane declare that the dissertation, which I hereby submit for the degree ***Magister Scientiae (Veterinary Science)*** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Ralengopeng Nick Mokotoane

30 / 05 / 2016

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List of abbreviations

AGID	agar gel immunodiffusion
AHS	African horse sickness
AHSV	African horse sickness virus
ARC-OVI	Agricultural Research Council-Onderstepoort Veterinary institute
ATP	adenosine-5'-triphosphate
bp	base pair (s)
BHK	baby hamster kidney
BLAST	basic local alignment search tool
BTV	bluetongue virus
°C	degrees Celsius
cDNA	complimentary DNA
CF	complement fixation
CO2	carbon dioxide
CPE	cytopathic effect
DAMBE	data analysis in molecular biology and evolution
DISC	disabled infectious single cycle
DMEM	Dulbecco's modified essential medium
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dsRNA	double-stranded RNA
EEV	equine encephalosis virus
ELISA	enzyme-linked immunosorbent assay

EtBr	ethidium bromide
FBS	fetal bovine serum
GIV	Great Island virus
HA	hemagglutinin
HBSS	Hank's balanced salt solution
HIV	human immunodeficiency virus
ICTV	International Committee on Taxonomy of Viruses
IFA	indirect immunofluorescence assay
M	molar
min	minute
ml	millilitre
mM	millimolar
NCBI	national center for biotechnology information
ng	nanogram
NGS	next-generation sequencing
NS	non-structural
OIE	World Organisation for Animal Health
ORF	open reading frame
OVI	Onderstepoort veterinary institute
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAse	ribonuclease
RT-PCR	reverse transcription PCR

RT-qPCR	reverse transcription quantitative PCR
S	segment
SCRV	Saint Croix River virus
sec	second
ssRNA	single-stranded RNA
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
$TCID_{50}$	50% tissue culture infectious dose
VIB	viral inclusion body
VN	virus neutralisation
VLP	virus-like particle
VP	viral protein
w/v	weight per volume
µl	microlitre

Summary

Complete genome comparison of the recent and historic field strains of African horse sickness virus isolated over four decades.

by

Ralengopeng Nick Mokotoane

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African horse sickness (AHS), caused by the African horse sickness virus (AHSV), is an economically important disease of equids. Outbreaks of the disease usually have a devastating effect on the susceptible equine populations. Due to the severity of AHS in horses, the economical impact and ability to spread rapidly, the disease has been listed by the World Organisation for Animal Health (OIE) as a notifiable disease. Nine serotypes of the virus have been identified. Transmission is mediated by biting midges of the genus *Culicoides*. Despite the fact that a cell culture-attenuated live virus vaccine is commercially available, AHS outbreaks occur annually in endemic regions. Several shortcomings of the vaccine itself and its application have been identified that may explain the occurrence of outbreaks.

In this study, we sought to evaluate the level of genetic divergence between the AHSV reference strains that the current polyvalent vaccine was based on and recent field isolates. The effectivity of the vaccine to induce complete protective immunity against current circulating strains is, thus, an additional concern and warranted further investigation. Similarly, the current serological diagnostic assays to detect virus or antibodies to the virus in the recent field isolates or horse sera respectively, are based on the reference isolates.

Towards accomplishing the above mentioned goal, a panel of recent AHSV field strains, isolated between 1994 and 2009, were selected for comparative analysis to the reference strains isolated in the 1960s. The complete, segmented double stranded RNA (dsRNA) genomes of nine recent field isolates of AHSV, representing serotypes 1 to 9, were characterised. Ultra-deep sequencing data of cDNA copies of the genome segments were generated. Consensus nucleotide sequences of each of the ten segments of each isolate were successfully assembled. Sequence data analysis showed that each virus isolate not only contained single serotype of AHSV, but was also free from other contaminating equine dsRNA viruses, such as equine encephalosis virus (EEV). The sequence data also confirmed the serotypes of the virus, as previously determined by virus neutralisation (VN) assays and reverse-transcription polymerase chain reaction (RT-PCR).

Intra-serotype comparative sequence analyses of the corresponding segments of the reference and recent field strains showed a maximum variability of 28% and 9% on nucleotide and amino acid level, respectively. The most variable genome segments were S2 encoding viral protein 2 (VP2), followed by S10 encoding non-structural protein 3 (NS3) and S6 encoding viral protein 5 (VP5). Therefore, these segments of the AHSV-9 reference and recent strains were further investigated. Comparative analysis of VP2 of the AHSV-9 reference strain with those of more recent field strains revealed a number of dissimilar amino acid mutations within, or adjacent to, known epitope-containing regions. In addition, significant changes were observed in the amino terminus. Some of the mutations correlated with altered predicted secondary structure and/or antigenicity profiles. Similar analysis of AHSV-9 VP5 indicated that the region between residue 101 and 201 was variable, although the overall predicted secondary structure appeared to be conserved. Our results indicated that the hydrophobic domain regions 1 (HD-1) and 2 (HD-2) respectively, in S10 (NS3), previously reported to play an important role in the function of NS3, remained conserved.

Chapter 1: Literature review

1.1 INTRODUCTION

African horse sickness (AHS) is an important disease of equids that affects susceptible horses severely with a mortality rate of up to 95% (Coetzer & Guthrie, 2004). AHS is an infectious, non-contagious, arthropod-borne viral disease. The aetiological agent of AHS is the African horse sickness virus (AHSV) (Vreede & Huismans, 1998). Due to the severity of AHS in horses, the economic impact and ability to spread rapidly, the disease has been listed by the World Organisation for Animal Health (OIE) as a notifiable disease.

1.2 HISTORY OF AHS RESEARCH AND IMPACT OF OUTBREAKS IN SOUTH AFRICA

AHS is endemic to sub-Saharan Africa (Henning, 1949) and a number of cases are reported annually, following a period of high rainfall during a summer season. More than 1500 cases of AHS have been confirmed in South Africa in the past four years, resulting in the death of 875 horses (www.racingsa.com). Although these figures are significantly lower than those reported in historical literature, they represent a significant loss of income in the horse industry and of livestock of the owners. Racing South Africa (www.racingsa.com) has reported that outbreaks of AHS and severe quarantine restrictions due to the disease have hampered the growth of foreign exchange income worth a potential R1 billion per annum.

AHS was first described in 1569 by Father Monclaro who was a missionary travelling from Portugal to East Africa and he made notes of a disease affecting horses transported from India into East Africa (Theiler, 1921). A description was given of a horse falling down when being led to the water and casting out yellow matter. This discharge is seen just before the horse succumbs to the pulmonary or acute form of the disease. It was, however, initially believed to have been as a result of horses ingesting poisoned grass. In 1719, the first major outbreak of the disease occurred in the Cape of Good Hope,

resulting in the loss of 1700 horses (Henning, 1949). Although this outbreak was followed by a number of other major and smaller outbreaks, the most devastating outbreak occurred in 1854 – 1855, resulting in the death of 70 000 horses (Henning, 1949). The horses lost due to the disease were believed to be more than 40% of the horse population at the Cape of Good Hope, estimated to be a loss of £525 000 (M'Fadyean, 1900; Theiler, 1921). This massive loss of horses led to an enquiry by authorities and the desire to find a remedy for the disease.

The disease itself was poorly understood and the causative agent was unknown. Local farmers and horse owners were, however, quick to realise the association between the dew on the grass in the morning and the outbreak of the disease when the horses were let out of their stables at that time (M'Fadyean, 1900; Vandenbergh, 2010). They associated this with malaria of some sort that was contracted by horses eating grass with dew. They also noticed that horses that were kept in high lying areas during an outbreak were less affected (Vandenbergh, 2010). Researchers debated whether the disease is contagious or not, but this possibility was ruled out by evidence indicating that cohabitation of healthy and diseased horses did not result in the development of symptoms in the healthy horses (Theiler, 1915).

M'Fadyean (1900) proved that AHS can be transmitted by subcutaneous inoculation with blood from affected animals. From that observation many considered the possibility that AHS can be transmitted by biting insects. M'Fadyean (1900) did not, however, believe that a bite from a vector was a common route of infection of horses. He concluded that, since the causative agent of AHS was filterable through Chamberland F filters and freely passed through the pores of a Berkefeld filter even in liquid containing albumen, the causative agent must be smaller than any of the known bacteria. Theiler (1915) referred to the causative agent as a filterable agent as nothing was known about the type of agent and its morphology, nor did it grow in the same media and conditions as bacteria known to them at the time. Although experimental evidence of insect transmission was lacking Theiler believed that the disease was communicated between animals by nocturnal biting winged insects (Theiler, 1915).

From what we know today at a much more advanced level of research into veterinary diseases, the conclusions of earlier researchers and farmers about the disease were not inaccurate. AHS is caused by a virus, the AHSV. A horse infected by AHSV usually displays one form of the disease, i.e. the horse sickness form, the cardiac or pulmonary forms, or the mixed form (both pulmonary and cardiac form) (Theiler, 1921). The dew on the grass in the morning is associated with the activity of the vector, *Culicoides imicola*, which plays a major role in the geographical distribution of the disease (Capela *et al.,* 2003).

1.3 HOST SPECIES OF AHS

AHS affects members of the *Equidae* family (Coetzer & Guthrie, 2004). African donkeys and mules are the least susceptible and rarely show any clinical signs of AHS, although viraemia may be present (Alexander, 1948; Fenner *et al.*, 1987). Horses are the most susceptible (Theiler, 1921; Coetzer & Guthrie, 2004). In zebras the disease is subclinical and as a result zebras are thought to play an important role in the transmission and the prevalence of the disease (Mellor & Hamblin, 2004). In addition to African donkeys, zebras act as reservoirs hosts (Alexander, 1948; Erasmus *et al.*, 1978; Hamblin *et al.*, 1998).

Theiler (1906) showed that dogs are also susceptible to experimental infection by AHSV and suffer similar symptoms as those seen in horses. Other researchers have however argued that neither wild, nor domestic dogs play a role in the transmission of the virus in nature (Haig & McIntosh, 1956; van Rensberg *et al.*, 1981; Alexander *et al.*, 1995). Work completed by Binepal (1992) supported these observations when he discovered that antibodies to all nine serotypes of AHSV were found naturally in zebras, horses, and elephants but not in sheep, goats and wild carnivores in east Africa. It has since been accepted that infection of dogs (wild or domestic) occurs as a result of these animals consuming AHSV infected horse meat and not through transmission by the arthropod vector. Nevertheless, more recently there has been a reported case of AHS in a domestic dog without a history of ingestion of horse meat (van Sittert *et al.*, 2013).

1.4 GEOGRAPHICAL DISTRIBUTION OF AHS

Three factors determine the expansion of the AHS-affected areas. These are the availability of susceptible animals, the presence of competent vectors that are able to transmit the virus and climate conditions favourable to the survival of the insect vectors (Mellor, 1993). Outbreaks of AHS occur throughout sub-Saharan Africa (Coetzer & Guthrie, 2004). Although the virus is endemic in the tropical and sub-tropical areas of Africa, epizootics have been recorded in northern Africa (Diaz-Montilla & Panos Marti, 1968), southern European countries (Diaz-Montilla & Panos Marti, 1967; Rodriguez *et al*., 1992) and the Middle East (Alexander, 1948). AHS has not been observed in the Western hemisphere, eastern Asia or Australasia.

Outbreaks of AHS in endemic regions are due to all nine serotypes of the virus, whereas serotype 9 is responsible for outbreaks in non-endemic regions (Mellor, 1993; Mellor & Hamblin, 2004). An exception to this was the 1987 - 1990 outbreaks in Spain which extended into Portugal and Morocco, for which AHSV-4 was isolated. It is believed that the importation of zebras from Namibia to the Safari park situated 50 km south west of Madrid was the origin of these unusually extended outbreaks outside the endemic regions (Lubroth, 1988).

1.5 TRANSMISSION OF AHSV

Several arthropod species and genera have been considered in the transmission of AHSV. *Culicoides* species are however identified as the primary vectors (du Toit, 1944*;* Wetzel *et al.*, 1970). In Africa, *C. imicola* and *C. bolitinos* transmit AHSV (Meiswinkel *et al.*, 2000; Venter *et al.*, 2000). *C. variipennis* can experimentally transmit AHSV, however, it is only dominant in the USA (Boorman *et al.*, 1975).

C. imicola is found in great numbers in the second half of summer, during warm and moist conditions (Becker *et al.*, 2012). They are most active from sunset to dawn, which is the period when transmission occurs (Becker *et al.*, 2012), via the intravenous rather than the

subcutaneous route (Henning, 1949). According to Capela *et al.* (2003), *C. imicola* has been found in abundance in areas affected by previous outbreaks of AHSV and bluetongue virus (BTV) in Portugal. As a result, the presence of this vector can serve as an early warning sign for possible outbreaks of AHS, taking into consideration factors like the climate (temperature, rainfall), population density of susceptible equines and movement of equines (Mellor, 1993).

C. bolitinos' abundance, unlike that of *C. imicola,* is independent of the soil type (Meiswinkel *et al.*, 2000). These vectors can be found in mountainous regions along with cattle, causing outbreaks in high-lying areas (Meiswinkel & Paweska, 2003). *C. bolitinos* inhabits and breeds in semi-moist cattle, African buffalo and blue wildebeest dung (Meiswinkel & Paweska, 2003).

No other arthropod species are implicated in the natural transmission of AHSV although AHSV has been isolated from mosquitoes. Their role in the transmission of the virus is however unclear (Mellor *et al.*, 1990). Ozawa & Nakata (1965) and Ozawa and co-workers (Ozawa *et al.,* 1966 a, b; Ozawa *et al*., 1970) have reported the transmission of AHSV to susceptible horses via experimentally infected *Anopheles stephensi, Culex pipiends* and mosquitoes of the genus *Aedes*, however transmission of AHSV by other researchers could not be demonstrated (Nieschulz *et al.*, 1934; Wetzel *et al.*, 1970). A number of workers reported the isolation of AHSV from ticks and the successful transmission of AHSV via tick bites to camels, dogs and horses under experimental conditions (Salama *et al.*, 1979, 1980; Awad *et al.*, 1981)

1.6 PATHOGENESIS AND CLINICAL DIAGNOSIS OF AHS

Following transmission of AHSV to horses, the virus replicates in the endothelium of the regional lymph capillary vessels and lymph nodes (Burrage & Laegreid, 1994; Coetzer & Guthrie, 2004; Mellor & Hamblin, 2004). The virus then enters the bloodstream where it associates with erythrocytes and white blood cells, and disseminates to the target organs, giving rise to primary and secondary viraemia, respectively. The duration of secondary viraemia in the organs of susceptible horses is between four and eight days, but not

commonly more than 21 days (Coetzer & Erasmus, 1994). The titre observed in horses may reach $10^{5.0}$ of 50% tissue culture infectious dose per millilitre (TCID50 virus/ml). However, in donkeys and zebras viraemia is lower ($<10^{3.0}TCID_{50}$/ml), but lasts longer, extending for up to four weeks (Mellor & Hamblin, 2004). Target organs include the lungs, large intestines and lymphoid tissues. Horses infected experimentally with AHSV show a high concentration of the virus within the spleen, lungs, caecum, pharynx, choroid plexus and most lymph nodes two days post-inoculation (Coetzer & Guthrie, 2004). Three days after inoculation, the virus can be found in most organs. The virus levels in the myocardium are, however, not higher than those observed in the blood. This indicates that the AHSV do not replicate in the myocardium, even in the cardiac form of the AHS disease (Coetzer & Guthrie, 2004). A high concentration of the AHSV in the lymphoid tissue results in lymphoid destruction, resulting in a low concentration of the lymphocytes (Coetzer & Guthrie, 2004).

Clinical diagnosis of AHS can be made based on known clinical signs of the disease and lesions (macro- and micro-lesions), the presence of competent arthropod vectors and available history of the movement of equines within and from enzootic areas (Ranz *et al.,* 1992). The clinical signs and lesions can be associated with one of the four disease forms i.e., the “pulmonary or dunkop” (“thin head”) form, “cardiac or dikkop” (“thick head”) form, the “horse sickness fever” form and the “mixed” or acute (both “cardiac” and “pulmonary” forms) form (Laegreid, 1994).

Horses affected by the “pulmonary or dunkop” (“thin head”) form of the disease can appear normal and healthy for hours until the onset of dyspnoea (shortness of breath or difficulty in breathing) and hyperpnoea (increased breathing rate). Prior to death the nostrils are flared, the mouth is open, the tongue protrudes, the neck and head are extended and heavy perspiration is noticeable (Rodriguez *et al.*, 1992).The most visible macrolesion associated with the pulmonary form is the interlobular oedema of the lungs and hydrothorax. This is coupled with the infiltration of sub-pleural and interlobular tissues, with a gelatinous yellowish exudate. The following may occur but are not observed in every pulmonary case: stabilised froth may be observed within the bronchial

tree, ascites in abdominal and thoracic cavities may occur and mucosa of the stomach may be hyperaemic and appear oedematous (Mellor & Hamblin, 2004).

Horses affected by the “cardiac” (“thick head”) form can have fever lasting for several weeks. In affected ponies fever is observed within four to six days post infection (Wohlsein *et al.*, 1997*).* Subcutaneous swellings of the neck, supraorbital fossae and head are seen from which the term dikkop is derived. Swellings of the eyelids, lips, cheeks, tongue and ventral thorax and abdomen are often present. The horse’s eyes are often partly closed due to the swelling. The respiratory rate increases slowly and breathing becomes more abdominal. Dyspnoea and cyanosis may supervene as a result of increased swelling. The most prominent macrolesions associated with the cardiac form of AHS are the presence of the gelatinous exudate in the subcutaneous, sub-fascial and intermuscular tissue and lymph nodes. Haemorrhages on the surfaces of the epicardium and endocardium are found and hydropericardium is observed. Petechial haemorrhages with or without cyanosis may occur on the serous membrane of the caecum and colon. Ascites may occur however with no observable oedema of the lungs (Mellor & Hamblin, 2004).

Horses affected by the horse sickness fever form may have a rectal temperature of 39 – 40ºC for one to six days. In some cases, short-lived slight conjunctivitis, loss of appetite, difficulty in breathing and an increase of the heart rate are seen. The mixed form AHS is usually the most common form, however, it is not often diagnosed in live horses. It is only when an autopsy is performed that the diagnosis can be made. Prior to the autopsy neither the pulmonary or cardiac form predominates, as such the diagnosis is referred to one of these forms (Rodriguez *et al.*, 1992). Horses affected by the mixed form can either show respiratory failure followed by oedematous swelling or alternatively show the cardiac form of AHS followed by respiratory difficulties. Macrolesions of the mixed form of AHS include lesions that are common to both the pulmonary and the cardiac forms.

Observed microlesions for horses affected by AHS are as a result of an increase in the permeability of the capillary walls and impairment in circulation (Mellor & Hamblin, 2004). Microlesions of the lungs include capillary congestion and serous infiltration of the interlobular tissues with enlargement of the alveoli. Enlargement of the liver’s central veins and the presence of the erythrocytes and blood pigments within the interstitial tissue

are observed. Fatty degeneration is observed within the parenchymous cells. Infiltration of the cells is observed in the renal cortex. Congestion is observed in the spleen and the intestinal and gastric mucosa. Swelling of the myocardium and skeletal muscles is observed (Mellor & Hamblin, 2004).

1.7 IMMUNE RESPONSE IN AHSV INFECTED HORSES

Owing to the high mortality rate in AHSV-infected horses, studies on the immunity of naturally or experimentally infected equines have been difficult to complete (Crafford, 2013). Nevertheless, immunity conferred by vaccination has been considered extensively in a number of studies on AHSV and BTV. A number of workers have shown that vaccination in horses result in detectable levels of neutralizing antibodies and that there is a strong correlation between the antibody titre and protection (Burrage *et al*., 1993; Chiam *et al*., 2009, 2001; Scanlen *et al*., 2002; Stone-Marschat *et al*., 1996; Martínez-Torrecuadrada *et al*., 1995). The neutralizing antibodies are directed against epitopes regions on VP2 and VP5. Infected and vaccinated horses are protected against homologous serotype challenges. There is therefore a good understanding of the humoral response to AHSV and BTV infection.

Pretorius *et al*. (2012) described the role of cellular immune response in a protective defence against AHS by studying the *in vitro* peripheral blood mononuclear cells (PBMC) recall immune responses against AHSV. In this study, PBMC collected from three horses vaccinated with viable attenuated AHSV-4, were stimulated *in vitro* with the virulent AHSV-4. Thereafter, the production of IFN-γ, other cellular and humoral cytokines and phenotype of the proliferating cells were determined using ELISPOT, quantitative PCR (qPCR) and flow cytometry, respectively. An increase in the B-cell population was observed in all three horses. Coincidently, virus-specific neutralising antibodies were detected and interleukin – 4 (IL-4) and IL- 6 cytokine mRNA synthesis was upregulated. An increase in AHSV-4 specific CD8+ T cells was also detected. This correlated inversely with the decrease in the CD4+ T cells. It is believed that the proliferating CD8+ T cells are cytotoxic to AHSV-infected cells.

1.8 CLASSIFICATION OF AHSV

AHSV belongs to the genus *Orbivirus* in the family *Reoviridae*, composing of mutli-segmented double stranded RNA (dsRNA) viruses. Currently 15 genera are included in the family (http://www.ictvonline.org/virusTaxonomy.asp). The prefix “orbi” in orbiviruses is based on the Latin word “orbis” meaning ring or circle and describes the characteristic capsomeric rings on the orbivirus core surface (Oellerman *et al.*, 1970; Borden *et al.*, 1971; Verwoerd *et al.*, 1972). Species in the genus *Orbivirus* all have a segmented dsRNA genome that is enclosed in an icosahedral protein capsid (Gorman, 1979). The genus *Orbivirus* includes 22 different serogroups among which are the AHSV and BTV (Mertens & Diprose, 2004). Orbiviruses are divided into groups based on their serological reactions hence the term serogroup. Viruses with common complement fixing antigens are grouped together given the name of the first isolate in that group (Gorman, 1979). Theiler (1915) was first to realise antigenic plurality of strains of AHSV. However, only after Alexander (1935a) propagated AHSV in mice brain, was further research on the antigenic types of AHSV completed. McIntosh (1958) used serum produced in rabbits and completed a neutralisation assay in mice of the rabbit antisera and virulent strain, in a study of 84 AHSV strains, to describe the existence of the seven immunological types of the virus. Using a similar approach, Howell (1962) identified two viral strains which formed two new groups of antigenic types. To date these previously described groups of the AHSV remain as the nine serotypes of the virus.

1.9 AHSV STRUCTURE AND GENOME

Extensive research has been completed on BTV, the prototype orbivirus. As such much of the understanding on the structure of orbiviruses, their replication in mammalian cells and other aspects of the viruses are based on the work completed on BTV and not repeated for AHSV as it is expected to be very similar. It is for this reason that discussions on the structure and replication (Section 1.11) are based on orbiviruses in general.

The AHSV genome comprises of ten linear, dsRNA segments (S1-10), numbered according to their migration rates through polyacrylamide gels (Verwoerd, 1969; Oellerman, 1970; Verwoerd *et al.*, 1970). These are further grouped according to their

size, i.e. small (S7-10), medium (M4-6) and large (L1-3). Certain segments of the genome contain conserved terminal hexanucleotide sequences. The 3' and 5' ends of the individual genome segments' RNA sequences show partial inverted complimentary. The existence of the inverted complimentary sequences suggests that single stranded RNA (ssRNA) species form secondary structures. The secondary structures may have a number of functional roles which includes protection of the viral messenger RNA (mRNA) species against exon digestion, extrusion of mRNA from the core following synthesis, replication and transcriptional regulation (Roy & Noad, 2006; Mertens & Dipprose, 2004).

In addition to coding for the structural and non-structural proteins, the AHSV genome also serves as a template for the synthesis of copies of the individual genome segments. With the exception of S9 and S10, each genome segment encodes a single protein (Table 1.1). S10 codes for the non-structural protein 3 (NS3) and 3A (NS3A) respectively, via a different in-frame translation initiation site (van Standen & Huismans, 1991; van Staden *et al.*, 1995). Firth (2008) reported the existence of a short overlapping coding sequence (CDS) in the orbivirus genome segment 9, overlapping with the VP6 encoding open reading frame (ORF) in the first (+1) reading frame. He observed that this overlapping coding sequence is present in all obiviruses with the exception of the Saint Croix River virus (SCRV). Belhouchet *et al.* (2011) and Ratinier *et al.* (2011) subsequently demonstrated the expression of the protein in cells infected with BTV and Great Island virus (GIV) and referred to it as NS4. The expression of two types of the NS4 (NS4-I and NS4-II) protein was demonstrated in both the cytoplasm and nucleus of AHSV-infected mammalian cells (Zwart *et al.*, 2015). NS4-I ORF is 435 nucleotides long encoding a polypeptide of 144 amino acids. The NS4-II ORF was found to be 465 nucleotides long, encoding a polypeptide of 154 amino acids in length (Zwart *et al.*, 2015).

Table 1.1: Functions of the proteins of AHSV and the genome segments encoding them.

Protein*	Protein size (kDa)	Location	Encoding genome segment	dsRNA genome segment length (bp)	Function and property in analogy to BTV
VP1	150	Subcore	L1	3965	Minor structural protein forming part of a transcriptionally active core. dsRNA synthesis from ss plus viral RNA strand.
VP2	124	Outer capsid	L2	3203	Virus entry into mammalian cells, Virus hemagglutinin activity. Virus egress.
VP3	103	Subcore layer	L3	2792	Highly conserved and important for the structure of the AHSV.
VP4	76	Subcore	M4	1978	Minor structural protein with RNA capping activity/capabilities. Guanylyltransferase, 5' RNA triphosphatase and NTPase
NS1	63	Non-structural protein	M5	1748	Forms tubules in the cytoplasm of infected cell Function unknown
VP5	56	Outer capsid	M6	1564	Membrane permeability, Virus entry.
VP7	38	Core surface layer	S7	1167	Plays a role in cell entry and infectivity of the core particles.
NS2	41	Non-structural protein	S8	1165	Associated with virus inclusion bodies. Binds ssRNA, NTPase activity. Phosphorylated
VP6	43	Subcore	S9	1169	Putative helicase, binds ssRNA and dsRNA
NS3/NS3 A	24/23	Non-structural protein	S10	758	Responsible for virus release by affecting membrane permeability.
NS4	17 - 20	Non-structural protein	S9	324 - 510	Function currently unknown

NS: non-structural protein, VP: viral protein. Data compiled from literature by Basak *et al.* (1997), Burrage *et al.* (1993), Boyce *et al.* (2004), de Waal & Huismans (2005), Grubman *et al*. (1990), Hassan & Roy (1999), Hassan *et al.* (2001), Maree & Huismans (1997), Martinez-Costas *et al.* (1998), Meiring *et al.* (2009), Ramadevi & Roy (1998), Stauber *et al.* (1997), van Niekerk *et al.* (2001), Vreede & Huismans (1998), Zwart *et al.* (2015).

The ultrastructure of the prototype *orbivirus* and related AHSV is complex and their assembly relies on highly specific interactions of the seven structural viral proteins (VP1 to VP7) in non-equimolar ratios. The VP3 layer is smooth and contains 120 copies of VP3 arranged as 12 dish-shaped decamers (Grimes *et al.* 1998). This layer serves as the

scaffold for 780 VP7 molecules (Fig.1.1), grouped as 260 column-shaped trimers in the outer core surface layer (Johnson & Speir, 1997). The characteristic appearance of the *orbivirus* core surface is attributed to the shape and ring-like arrangement of VP7 trimers as hexamers or pentamers. The interactions between the inner and outer core layer are extensive (Limn & Roy, 2003). The three minor core proteins, VP1 (10 or 12 copies), VP4 (20 or 24 copies that form dimers) and VP6 (60 or 72 copies that form hexamers) not only interact with each other, but are also attached to the inner surface of VP3, below the five-fold axis (Fig.1.1) (Stuart & Grimes, 2006). The outer layer of the virion is composed of 360 VP5 molecules arranged as 120 globular-shaped trimers and 180 VP2 molecules organised as 60 propeller-shaped triskelion-type motives. VP2 is the most surface-exposed protein in the virion (Nason *et al.*, 2004)

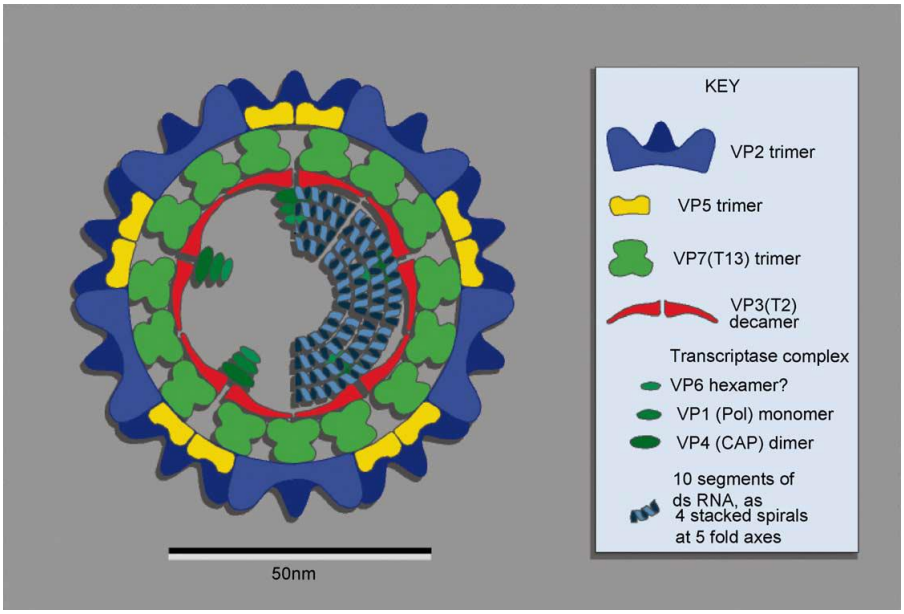

Figure 1.1: Schematic diagram representing the structure of the BTV particle (Mertens & Diprose, 2004)

1.10 LABORATORY DIAGNOSIS OF AHS

Laboratory diagnosis of AHS is required, since most of the clinical signs and lesions associated with the disease can be confused with other equine diseases. In addition, several characteristics of the epidemiology of the viruses that cause AHS and EEV are

similar (Mellor & Hamblin, 2004). To successfully identify the pathogen during laboratory testing, virus isolation is traditionally performed (Ranz *et al.*, 1992; Laegreid, 1994). Blood of infected animals is collected during the febrile stage as the preferred *ante mortem* sample (Hazrati *et al.*, 1973; Laegreid *et al.*, 1993). Following the death of the animal, the lung, spleen and lymph node are chosen as tissue samples for the isolation of AHSV. Monolayers of the African green monkey kidney (Vero) or baby hamster kidney cells (BHK) are then infected with the suspensions prepared from the blood or tissue samples. The infected cells often show cytopathic effects (CPE) between the third and seventh day (Laegreid, 1994). CPE may not be visible from the first passage and several 'blind' or subsequent passages may be required to increase the virus titre. As a result, intracerebral inoculation of neonatal mice, which are between two to four days old, is the preferred method for primary isolation of the virus. With intracerebral inoculation the neonatal mice are observed for possible neurological clinical signs. Following intravenous inoculation of embryonated hen's eggs, the AHSV is capable of adapting and replicating in the eggs (Boormans *et al.*, 1975).

Techniques to detect viral antigens or viral nucleic acid in infected sample have also been developed. Such assays include serogroup-specific tests which target the viral antigens and conserved genome segments and, serotype-specific tests which target genome segment 2 and its encoded protein, VP2. VP1, VP3, VP7, the non-structural proteins (NS1-4) of the AHSV and the genome segments encoding them are routinely targeted in serogroup-specific assays. The tests that can be utilised to identify AHSV include, RT-PCR (Aradaib, 2009), real-time PCR (Fernandez-Pinero *et al*., 2009), RT-qPCR (Guthrie *et al.*, 2013), real time RT-qPCR (Quan *et al*., 2010), complement fixation (CF) (Mcintosh, 1958), enzyme-linked immonusorbent assay (ELISA) (Williams, 1987) and the direct and indirect immunofluorescence assay (IFA) (Huq & Ansari, 1961). Serogroup antibody detection can be completed using CF, ELISA and agar gel immunodiffusion (AGID) (Hazrati *et al.*, 1968). Brown *et al.* (1994) also described digoxigenin labelled RNA probes for serogroup-specific detection of the AHSV.

Serotype-specific assays used for AHSV are able to distinguish between the nine different serotypes of the virus. These includes the virus neutralisation (VN) assay (Howell 1962;

Hazrati & Ozawa, 1965) and the RT-PCR (Sailleau *et al.*, 2000). The use of ^{32}P labelled probes developed from cloned full-length VP2-genes of the reference strains of the AHSV, has been described for serotype-specific detection of AHSV (Koekemoer *et al.*, 2000). Serotype-specific antibody detection can be completed using the VN test (Blackburn & Swanepoel, 1988).

1.11 ORBIVIRUS REPLICATION AND MORPHOGENESIS

BTV replication initiates with VP2 located on the virion surface mediating adsorption and internalisation in mammalian cells (Huismans & van Djik, 1990; Hassan & Roy, 1999). Cowley & Gorman (1987) demonstrated that VP2 displays hemagglutination activity which enables the virion to bind to the membrane of susceptible cells. The bound virus is then internalised into endosomes via a receptor-mediated endocytotic pathway, involving the formation of clathrin-coated vesicles that fuse to the endosomes (Fig.1.2) (Forzan *et al.*, 2004). Acidity within the endosomes, triggers removal of VP2 and VP5 proteins which play a role in the fusion of the virus particle to the endosomal membrane, as well as the permeabilisation. The virus core particle is subsequently released into the cytoplasm (Fig.1.2).

The release of the core particles into the cytoplasm activates the transcription complex (VP1, VP4, and VP6) (Mertens & Dipprose, 2004). The VP6 protein, which has been shown to exhibit helicase activities, unwinds dsRNA within the core allowing for transcription to be initiated (Stauber *et al.*, 1997; De Waal & Huismans, 2005). The transcription process within the core involves the VP1 protein (RNA dependent RNA polymerase) which produces mRNA transcripts (Vreede & Huimans, 1998; Boyce *et al.*, 2004). According to Boyce *et al. (*2004), BTV VP1 binds to ssRNA to synthesise dsRNA. VP4 protein within the core ensures 5' terminal capping of the synthesised mRNA transcripts (Ramadevi & Roy 1998; van den Bout, 2005). Ten capped transcripts are then released from the core into the cytoplasm which enables synthesis of seven structural proteins and the four non-structural proteins. The 5' capped mRNA transcripts, released into the cytosol also serve as template for the synthesis of the dsRNA genome segments and the viral proteins (Roy & Noad, 2006). At least two hours after viral infection the first

virus-specific proteins are detectable, and synthesised continuously in infected mammalian cells, till cell death (Huismans, 1979).

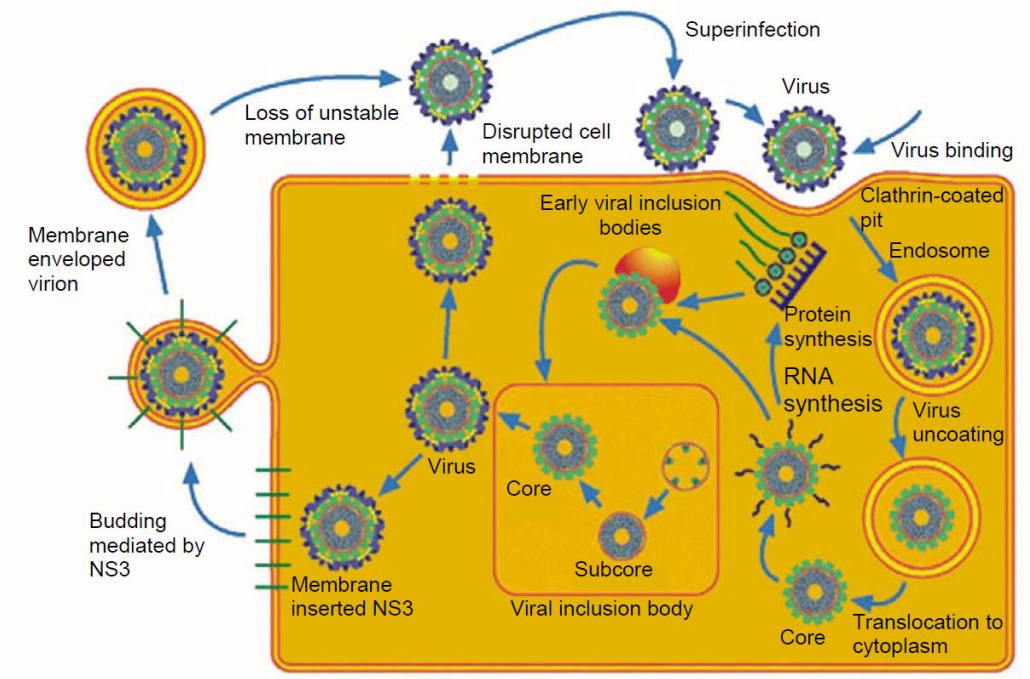

Figure 1.2: Schematic diagram representing the BTV replication cycle (Mertens *et al*., 2004) depicting virus binding and entry, uncoating and formation of replicative complexes, formation of virus-encoded tubules and inclusion bodies, translocation of progeny viruses to the cell surface and virus exit.

Soon after translation initiation, the viral cores become associated with the matrix which over time surrounds the cores to form the virus assembly factories or virus inclusion bodies (VIB) (Fig.1.2).The VIBs increase in size and numbers as infection of the cells progresses. These VIBs are a centre for BTV replication and early viral assembly, therefore, it contains the viral messenger RNA and proteins (Eaton & Hyatt, 1989; Brookes *et al.*, 1993). Assembly of the VIBs for BTV is controlled by NS2 (Thomas *et al.*, 1990). In the presence of phosphorylated NS2 protein, VIBs are formed. NS2 has also been shown to have high affinity for ssRNA (Thomas *et al.*, 1990). This gives a good explanation as to how plus sense ssRNA are selected from a pool of mRNAs, to be included into the VIBs. Regions within the genome segments which enable the individual segments to bind to NS2 as ssRNA, are those which form hairpin-loop secondary

structures (Lymperopoulos *et al*., 2003). Until recently, there has been a poor understanding as to how single copies of the individual genome segments are included into newly formed core particles. According to Sung & Roy (2014), for BTV and other viruses in the family *Reoviridae*, ssRNA segments of the viral genome form an RNA complex prior packaging. The formation of this complex is initiated by the smallest segments, S10, encoding for NS3 and NS3A. S10 interacts with other smaller segments (S7, S8 and S9) through its 5' and 3' untranslated regions (UTRs), forming a complex which recruits other segments. It is likely that several such complexes are formed which combine prior to packaging (Sung & Roy, 2014).

In the presence of the synthesised viral proteins and ssRNA within the VIBs, progeny viral assembly ensues with VP1 and VP4 proteins forming a stable complex (Nason *et al.*, 2004). This complex is simultaneously associated with the VP3 decamers, which are suggested to be the first stable assembly intermediates of the VP3 layer (Fig.1.2) (Kar *et al.*, 2004). During the assembly of the subcore, the genomes of the viruses form around the VP4 and VP1 complex. VP7 proteins then form trimers which are important for viral core assembly. These VP7 trimers are suggested to initially form strong contacts with the VP3 scaffold at a number of sites, followed by weaker interactions to complete the formation of the outer layer of the core (Roy & Noad, 2006). It is unclear as to how and when the VP6 protein is assembled into the subcore (Roy & Noad, 2006).

Following formation of the core, the VP2 and VP5 proteins are added independently to the VP7 layer (Nason *et al*., 2004). These proteins do not interact with the underlying VP7 layer but rather the triangular top portion of VP7 in order to form the outer capsid. Knowledge on where about in the cell the VP2 and VP5 proteins are added to the VP7 layer is limited.

The progeny virus is then released from the infected mammalian and insect cells, by the involvement of the NS1 and NS3 proteins. NS1 is abundantly expressed in BTV-infected cells and is observed to form microtubules within the cytoplasm (Owens *et al.*, 2004). The expression levels of NS3/NS3A proteins is low in BTV-infected mammalian cells and high in BTV-infected insect cells (French *et al.*, 1989; Guirakhoo *et al.*, 1995). It is suggested that cellular pathogenesis of BTV-infected cells is determined by the relative ratio of the

levels of NS1 tubules to those of the NS3 protein within the cytoplasm of the infected cells. When NS1 levels are high relative to NS3 protein levels, as in the case of mammalian cells, lysis and cell death are observed. Conversely, NS3-directed nonlytic virus release is observed as with the insect cells (Owens *et al.*, 2004). The nonlytic release of BTV is suggested to occur through interaction of the NS3 protein to cellular protein p11 and Tsg 101 as well as viral VP2 protein (Beaton *et al.*, 2002; Roy & Noad, 2006; Wirblich *et al.*, 2006). The lytic release of the BTV involves the NS3 protein functioning as a viroporin, causing the membrane of the host cell to be permeable (Han & Harty, 2004). The permeabilisation of the membrane subsequently causes a pore through which virus particles are extruded without a lipid membrane being acquired.

1.12 PREVENTION AND CONTROL

As a result of the devastating loss of horses in the 1800's, the disruption of normal daily use of horses and the economic impact thereof, many farmers and owners were prompted to desperately seek a cure for AHS. Local authorities, therefore, offered a reward of £25 000 to any individual who could find a cure for AHS and other animal diseases. A number of ineffective approaches were then proposed and/or explored (Vandenbergh, 2010). Nevertheless, to date there is no specific treatment for animals affected by AHS apart from giving them good rest and good husbandry. Horse owners discovered earlier on, however, that stabling from dusk to dawn control and reduce the incidence of AHS. To date, stabling is considered to be an effective alternative method of control due to the exophilic characteristic of most *Culicoides* species, including the primary vectors of AHSV in Africa (Meiswinkel *et al.*, 2000). Horses must be kept in inserts-free stables before sunset until a few hours after sunrise. In addition to stabling, the number of infective insert bites to the susceptible horses can be influenced by vector control. The control of vectors include recognising and destroying their breeding sites, the use of systemic insecticides toxic to the immature stages of *Culicoides,* however, of low toxicity to mammalians. Effective only for a few hours after being applied to animals, repellents can be used to reduce the biting rates of the *Cullicoides* species (Mellor & Hamblin, 2004).

Following epizootics of AHS in the Middle East, which resulted in the loss of 300 000 equines (Rodriguez *et al.*, 1992), AHS became a world concern and, hence, has been listed as a notifiable disease by the OIE. In 1984, AHS became a state-controlled disease in South Africa (Animal disease act No. 35 of 1984). The Western Cape Province is divided into two areas for control of AHS, the AHS endemic area and control area (Bosman *et al.*, 1995; Sinclair *et al.*, 2006; www.racingsa.com). The AHS control area is divided into three zones, i.e. the AHS-free zone, the surveillance zone and the protection zone (Fig.1.3). The free zone has historically been free of AHS, whereas the surveillance zone serves for early warning (Sinclair *et al.*, 2006). Both the surveillance and the free zones are crucial to South Africa retaining its export status and ability to export equines into and out of the country. As a result, strict movement controls into or between the zones are in place and are to be adhered to (Sinclair *et al.*, 2006; Western Cape Department of Agriculture, 2014). The movement of horses from an infected zone into the control area requires a passport and movement permit showing that the horse has been vaccinated according to the specified guidelines (Western Cape Department of Agriculture, 2014). The movement of unregistered equines (i.e. unvaccinated donkeys, mules and zebras) into the control area is permitted only for the months of July and August, provided they these and other requirements are met, i.e. the equine shows no clinical signs, applicable laboratory tests are completed and it is under quarantine for at least 40 days prior to departure. Strict quarantine and testing procedures are also followed prior to exportation of the horses from South Africa (Western Cape Department of Agriculture, 2014). In non-endemic areas, viraemic horses may be slaughtered early in an epidemic to minimise transmission (Rodriguez *et al.*, 1992).

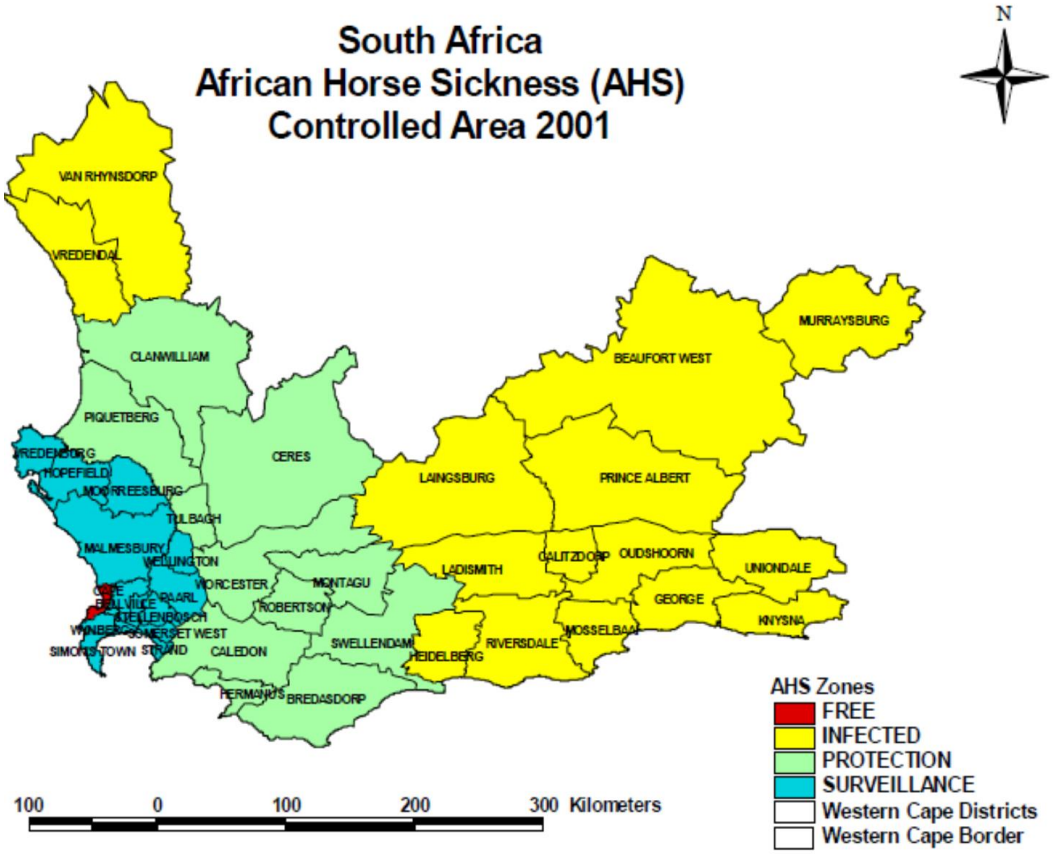

Figure 1.3: The map shows the AHS control area in the Western Cape Province in South Africa that is divided into the protection, surveillance and free zones (Western Cape Department of Agriculture, 2003). The control area is surrounded by the AHS-infected zone.

1.11.1 AHSV-antisera mixtures

Vaccination of horses in AHS endemic areas remains the most effective method for the control of AHS (van Dijk, 1998; Mellor & Hamblin, 2004; Patel & Heldens, 2009). In the 1900's, Theiler (1908, 1909) reported the first immunisation strategy for AHS by inoculating susceptible horses with a mixture containing both a virulent AHSV strain and hyper immune horse sera against the same strain. This regime was continued until 1933, with some modifications, but discontinued thereafter as a result of vaccine related deaths (2 - 10%), limitations in the amount of hyper immune sera that could be produced, and the necessity for repeated inoculation (Henning, 1949).

1.11.2 Inactivated vaccines

Ozawa & Bahrami (1966) described the experimental vaccination of horses using formalin inactivated AHSV-9. Horses vaccinated with the inactivated AHSV-9 developed protective immunity against the homologous strain of the virus, five weeks post vaccination. An inactivated (formalin-inactivated) AHS vaccine made from AHSV-4 became commercially available in Europe in 1990 although it is not in production today (Dubourget *et al.*, 1992).

The efficacy of the inactivated AHSV-4 vaccine using the one dose regimen was evaluated in nine ponies, six of which did not show any clinical signs of AHS following a challenge-inoculation with the virulent strain of the virus. Overall, this vaccine was shown to be protective against the virulent strains of AHSV even with mild side effect (House *et al.*, 1992).

In a follow-up study on the efficacy of the vaccine using a two dose-regimen, five ponies were vaccinated and thereafter challenged with the virulent strain of AHSV-4. All ponies showed no clinical signs or any anamnestic response. However, one pony had detectable viraemia (House *et al.*, 1994). The viraemia is believed to be as a result of the virus present in the challenge inoculation and not due to a replication of the virus in the host.

Inactivate vaccines have their drawbacks, one of them being that they require an adjuvant (an additive to the vaccine which is not antigenic, but assists in enhancing immunogenicity) to induce a strong immune response (Ward, 2011). Inactivated vaccines require more than one round of vaccination for full protection (House *et al.*, 1994) and often this is not long lived (Ward, 2011). As a result booster doses are needed. Inactivated vaccines are expensive to manufacture and there is a risk of incomplete inactivation.

1.11.3 Attenuated vaccines

In the 1930s, neurotropic AHS attenuated live virus vaccines were developed and used for almost four decades in South Africa and for epizootics of AHS in the Middle East, Egypt and Israel. This vaccine was prepared by serial (100x) intracerebral passage of

AHSV field isolates in mice (Nieschulz, 1934; Alexander, 1935b). Multiple serotypes of virulent viruses isolated from naturally infected horses (viscerotropic strains) were attenuated. Although a protective immune response was induced with some of the vaccines, those against other serotypes were not effective while other attenuated strains produced severe clinical signs in susceptible horses (Alexander *et al*., 1936). This related to the number of intracerebral passages required for each serotype. A number of negative reports on the effect of the vaccine were however reported. Pavri & Anderson (1963) reported the isolation of AHSV vaccine strain from the brains of two horses vaccinated with polyvalent attenuated vaccine of the virus. Five cases of mules turning blind following vaccination were also reported (Alexander *et al.*, 1936).

With an increase in the demand of neurotropic AHS attenuated live virus vaccine, and a limited supply of mice, it became clear that there was a need to produce the vaccines using an alternative method. Based on work completed by Woodruff & Goodpasture (1931) and Goodpasture *et al.* (1932), Alexander (1938) was able to propagate a neurotropic strain of AHSV in the brain of a chicken embryo while multiplication of the virus in the membrane and body was not observed. Although the number of embryonated Hens' eggs required to prepare a minimum vaccine dose were fewer than the number of mice required, the approach was more laborious.

Production of the first polyvalent cell cultured-attenuated AHSV live virus vaccine was made possible by the ability to propagate viruses in cultured mammalian cells (Enders *et al.*, 1949). Erasmus (1963) successfully propagated AHSV in BHK cells. He showed that large plaque variants were non-pathogenic (avirulent) (Erasmus, 1973), which led to subsequent development of polyvalent vaccines currently used for AHS. The vaccine is sold in two vials containing AHSV serotypes 1, 3 and 4 and 2, 6, 7 and 8, respectively (Onderstepoort Biological Products, South Africa). The vaccine composition prior to 1990 also contained serotype 5. However, following reports of severe reactions and deaths, post animal vaccination season of 1990, all AHS attenuated vaccines with the composition inclusive of serotype 5 were discontinued. It was discovered that serotype 4 and 5 strains included in the vaccine were responsible for the observed reactions and deaths of weanlings, possibly due to incomplete attenuation of the strains or interference

of the strains included in the first vial (Erasmus, 1994). However, following further attenuations of the serotype 4 strains and including it into the vaccine, safety and efficacy of the vaccine was improved. Serotype 9 of the virus is not included in the vaccine as it is considered not to be responsible for outbreaks of AHS in the southern Africa. Serotypes 5 and 9 are still not included in vaccine manufactured of AHS also due to putative cross protection offered by serotypes 8 and 6, respectively (von Teichman *et al*., 2010).

Although cell culture-attenuated live virus vaccines have been used successfully in endemic situations, there are a few concerns associated with their use in epidemic situations. There are no cell culture-attenuated live virus vaccines licensed for use in the EU. South African manufactured live attenuated vaccine are standardised, not allowing for manufacturing of other monovalent and polyvalent vaccines appropriate for the specific outbreaks. This may cause a delay in response to certain emergent situations making it impossible/difficult to respond to the incursions. Although not proven yet there is a concern that a live attenuated vaccine may act like a teratogen when used on pregnant mares (Mellor & Hamblin, 2004). There is difficulty in immunising young animals due to passive maternal immunity. Using the South African manufactured AHS live attenuated vaccines elsewhere in the world allows introduction of new viral topotypes. Incomplete attenuation of virus can cause viraemia in inoculated animals which could lead to transmission. Vaccine strains can exchange their genome segments (reassortment) with wild type viruses resulting in new genotypes. Virions presenting the new genotypes may exhibit enhanced virulence and new antigenic properties (Mellor & Hamblin, 2004).

1.11.4 New generation vaccines

Due to these concerns associated with the current AHS cell-attenuated vaccine, attempts to develop new vaccines have been made. DNA vaccines (Romito *et al.*, 1999), subunit vaccines (Roy *et al.*, 1996) and live viral vector vaccines (Stone-Marschat *et al.*, 1996; Guthrie *et al.*, 2009; El Garch *et al.*, 2012), co-expressing genes encoding proteins with the antigenic region of the virus have been described, however, none are available commercially for AHS.

It has been suggested that by developing safe and effective subunit and inactivated vaccines some of the problems associated with live attenuated viruses could be avoided. Additionally, subunit and inactivated vaccines have some important advantages, such as DIVA (differentiating infected from vaccinated animals) properties which enable the development of specific diagnostics assays to differentiate between vaccinated and naturally infected animals (House *et al.*, 1994; Minke *et al.*, 2004a; Pasick, 2004; Maclachlan *et al.*, 2007; Meeusen *et al.*, 2007). This is particularly important for incursion of AHS into areas previously identified to be free of the disease. Since these vaccines are non-replicative, the risk of transmitting the vaccine strain is eliminated (Mellor & Hamblin, 2004).

Unlike inactivated vaccines, VP2 subunit vaccines are less expensive. They presents a promising alternative to live attenuated vaccines. Experimentally used subunit vaccines for AHSV are synthesised using a baculovirus expression vector which is modified to express the outer capsid proteins of the virus. The use of baculovirus-expressed AHSV-4 VP2 has been shown to be protective against the virulent homologous strain of the virus (Roy *et al.*, 1996). However, conflicting results have also been reported. In a similar study, Martínez-Torrecuadrada *et al.* (1996) observed that full protection against the virulent AHSV-4 required a combination of the baculovirus-expressed VP2, VP5 and VP7 viral proteins. The failure of the baculovirus-expressed VP2 to induce a protective immune response, in the latter study, has been attributed to the differences in the conformation of the VP2 protein expressed *in vitro* compared to the *in vivo* expressed VP2 in the presence of other viral proteins. In BTV, the immunogenicity of the baculovirus-expressed VP2 has also been shown to be enhanced when co-expressed with the VP5 protein (Roy *et al.*, 1990).

It was discovered that the majority of the baculovirus-expressed AHSV-5 VP2 proteins in the crude cell lysates were present in insoluble aggregated form. VP2 in its insoluble form failed to induce a protective immune response in horses (du Plessis *et al.,* 1998). Scanlen *et al.* (2002) showed that a soluble baculovirus-expressed VP2, not co-expressed with VP5 or the inner core proteins was sufficient to induce protective immunity in horses. However, full protection by a subunit vaccine against its corresponding serotype required

the right adjuvant. This indicates that the type and dose level of the adjuvant used determines the efficacy of the subunit vaccine (Scanlen *et al.,* 2002).

In BTV, the outer capsid proteins (VP2 and VP5) expressed with the inner core proteins (VP3 and VP7), to form the virus-like particles (VLPs) have been shown to induce protective immunity in sheep, however with mild clinical reaction and detected viraemia (Roy *et al.*, 1992). Although VLPs lack the genetic material found within the authentic virion their configuration mimic that of the authentic virion. Experimental vaccination of sheep with core-like particles (CLPs) resembling the native viral cores, have also been described for BTV (Roy & Sutton, 1998). CLPs are formed by co-expression of the VP3 and VP7 proteins in insect cells using a dual or multigene baculovirus expression system. Using the CLPs partial immunity was observed in sheep against a virulent challenge. Both the CLPs and VLPs in experimental vaccination of animals take to advantage the capacity of the structural proteins to self–assemble and form virus-like particles, as observed during orbivirus replication, and that of the baculovirus expression vector to accommodate large amounts of foreign DNA (French *et al.*, 1989; Roy & Sutton, 1998).

Essentially VLPs and CLPs are an extension of VP2 and multi-protein subunit vaccines therefore the disadvantages/limitations experienced with these vaccines are similar to those described for VP2 and multi-protein subunit vaccines. Repeated immunisation is required for long lasting immunity in the animals. Although immunity was observed for sheep vaccinated with the BTV-VLPs without an adjuvant, the use of an oil based adjuvant further enhanced the immunity (Roy *et al.*, 1992). CLPs were also used together with the adjuvant to induce the partial immunity observed in experimentally vaccinated sheep (Roy & Sutton, 1998). Scanlen *et al.* (2002) has indicated the importance of using the correct dose and the correct adjuvant in ensuring that complete immunity is induced by the VP2 subunit vaccine. Despite significant research progress shown with the VLPs, CLPs and VP2 subunit vaccines, none have been produced for commercialisation purposes, possibly due to difficulties in large-scale production (Mellor & Hamblin, 2004).

Other vaccination strategies have therefore been explored which include the use of recombinant virus-vectored vaccines. The latter vaccines are genetically modified to express one or more recombinant viral proteins known to be immunogenic (Ward, 2011).

For AHSV, pox viruses have been used experimentally as vectors for the expression of the VP2, VP5, VP7 and NS3 proteins. Although no such vaccine has been produced commercially for AHS, licensed commercial recombinant virus-vectored vaccines, have been produced for other equine diseases (Poulet *et al.*, 2003; Minke *et al.*, 2004b; Poulet *et al.*, 2007).

Stone-Marschat *et al.* (1996) reported that the use of VP2 (in vaccinia-L2 Vero lysate) alone was sufficient to protect immunised horses against a challenge inoculation of the corresponding strain of the virus. In his work a recombinant vaccinia virus which expressed VP2 of AHSV-4 was prepared. Horses vaccinated with the construct were protected against the virulent homologous strain of the virus. Chiam *et al.* (2009) reported immunogenicity in ponies that have been vaccinated with recombinant modified vaccinia Ankara viruses, expressing VP2, VP7 and NS3 respectively. Although a humoral response against the vaccine was detectable, inflammation of the inoculation site and the elevated temperature were also observed for the first inoculation of the ponies.

Guthrie *et al.* (2009) has reported the development of a recombinant canarypox virus vector co-expressing the outer capsid proteins (VP2 and VP5) of AHSV-4. All horses vaccinated showed no adverse reaction to the vaccine and had solid immunity against the virulent AHSV-4. The detection of VP2 and VP5 specific IFN-γ responses, assessed by enzyme linked immune spot (ELISpot), showed that vaccinated horses developed a notable IFN-γ production in comparison to unvaccinated horses (El Garch *et al.*, 2012).

The recent successful application of reverse genetics to orbviruses has given a possibility of creating vaccines which do not replicate in host cells (none complementing cell line) and do not need multiple dosing for full protection (Matsuo *et al.*, 2011). A disabled infectious single cycle (DISC) vaccine has been described for BTV, designed by a major deletion in the coding region of VP6 (RNA replicase) and the introduction of a marker gene. Although these vaccines are not attenuated nor inactivated they contain the positive characteristic features of live attenuated, inactivated and subunit vaccines, but at the same time addresses the concerns with the use of each one of them. DISC vaccines replicate only once in the host cells therefore there is no concern over incomplete attenuation leading to viraemia, no genome reassortment can occur. A single dose of

BTV vaccine has been shown to prevent the replication of the virulent viral challenge in sheep thus overcoming the challenge faced with inactivated and subunit vaccines which require multiply dosing and large quantities of the vaccine to elicit an immune response (Matsuo *et al.*, 2011). Similar to subunit vaccines, DISC vaccines present to the host immune system the antigen to trigger an immune response, however, no adjuvant is needed for DISC vaccines. Multivalent cocktail vaccination with DISC viruses has been described for which six different serotypes of BTV were used simultaneously to inject sheep and confer protection (Celma *et al.*, 2013). DISC vaccines have thus become a promising alternative to the already available cell culture-attenuated live virus vaccine and ongoing research and development into subunits and inactivated vaccines.

1.13 GENETIC VARIATION AND VACCINATION

High mutation rate in RNA viruses has been reported. It is reported to be up to a 100 fold more than that observed in DNA viral genomes. This is due to the fact that the RNA polymerase lacks the 3' to 5' proof reading activity (Domingo *et al.*, 1996). As a result RNA viruses' high mutation rate with occurrence of recombination and genome re-assortment in segmented genomes means the emergence of new genotypes resulting in a complex and dynamic group of viruses referred to as viral quasispecies (Domingo & Holland, 1997). The term quasispecies was described by Eigen & Shuster (1979) which in its original description, refers to a population of mutants which are similar for most part, to the consensus sequence or average sequence of the population. Viral quasispecies are good for viral fitness, however, can pose a problem for the control of RNA viruses causing human and animal diseases. A number of such diseases caused by RNA viruses include human immunodeficiency virus (HIV), foot-and-mouth disease, equine influenza, AHS and many others.

The high mutation rate of RNA viruses may lead to antigenic changes which subsequently result in the new variants escaping the immune response (Boni, 2008). This may render the vaccine as being ineffective and requiring an update (Carrat & Flahault, 2007). Studies have been completed on RNA containing viruses in other genera and families

indicating a significance of new variants on vaccine and vice versa. A study on equine influenza A indicated that a single amino acid substitution was responsible for the antigenic drift (change in the antigenic region of the virus) of subsequent strains from the vaccine strains (Lewis *et al.*, 2011).

Most viruses in the orbivirus genus are arthropod transmitted RNA viruses and as a result evolve more slowly than non-arthropod transmitted RNA viruses (Scott *et al.*, 1994; Weaver *et al.*, 1992). It has been suggested that the slow rate of evolution is due to compromise by the virus to replicate both in the insect vector and mammalian host which both represent two different environments. By replication in the host and vector, the virus is kept in overlapping fitness peaks of the two environments however restricted to little evolutionary change (Novella *et al.*, 1999).

Evolution of the BTV genome due to genetic drift, reassortment and intragenic recombination has been described (Carpi *et al.*, 2010). Segments of the BTV genome evolve independently of each other by genetic drift in a host specific fashion resulting in quasispecies populations in the host vertebrate and the vector (Bonneau *et al.*, 2001). Reassortment in BTV is responsible for genetic shift, this occurs as a results of co-infection of different strains of the virus or different serotypes into one host or vector. Intragenic recombination for BTV has been demonstrated whereby mosaic genes are generated from splicing of homologous genes from different ancestral viral strains (Carpi *et al.*, 2010; Niedbalski, 2013). It has been demonstrated that evolution and random fixation of quasispecies variants, during transmission between host and vector, results in differences observed between field isolates of the same serotypes for BTV (Bonneau *et al.*, 2001). Such genetic variation has also been observed for AHSV (du Plessis & Nel, 1997; van Niekerk *et al.*, 2001; Huismans *et al.*, 2004). It is thus evident that AHSV also does evolve, even though its evolutionary dynamic has not been investigated as is the case with BTV.

It is important to be aware of the changes in the virus that can accumulate over time particularly for vaccine development and diagnostics. Due to great similarity in their structure and how they are transmitted there is a great possibility that AHSV like BTV may evolve by reassortment and recombination in the field. There is therefore a possibility that

antigenically variants strains of the AHSV may emerge overtime making it necessary to determine the antigenic and genetic composition of the predominant viral strains.

1.14 WHOLE GENOME SEQUENCING AND APPLICATIONS

Next-generation sequencing (NGS) has changed the field of comparative genomics resulting in the witnessed increase in studies comparing genomes or genes of individual organisms. This is also evident in the continuing effort to improve and develop new analytical tools. NGS is an advance in sequencing technology from Sanger sequencing (Sanger *et al.*, 1977), resulting in the capability of sequencing 1-7 kilobases at a time to a massively parallel high-throughput sequencing of a whole genome at a lower cost. With NGS, a large volume of sequence data is generated over a relatively short period of time and provides in-depths nucleic acid data of the sequenced genome, hence the term 'ultra-deep' sequencing (Mardis, 2008; Reis-Filho, 2009).

Comparative genomics is used extensively in biological research as a means of identifying new genes, determining the relationships between organisms and identifying the differences between individuals. The outcome of the analysis enables appropriate strategies for disease control and vaccine development, among others (Eaton & Gould, 1987; Pellegrini *et al.*, 1999; Chain *et al.*, 2003). Although a comparative genomics study can be completed in a number of ways, genome sequences are often aligned with bioinformatics software tools appropriate analysis tools to analyse the differences and similarities between genes or segments of interest and to study numerous other aspects of the genes/genomes and deduced amino acid sequence. From the alignments gene function(s) can be inferred and evolutionary relationships or epidemiology determined by phylogenetic analysis (Ganesan, 2009).

Using comparative genomics, researchers working on BTV and other orbiviruses have been able to characterise newly identified viral species belonging to the orbivirus genus (Attoui *et al.*, 2001; Belaganahalli *et al.*, 2011). There have also been reports on the use of comparative genomics in understanding the evolutionary mechanisms of the genomes in orbviruses (Stott *et al.*, 1987) and other viruses (Holmes, 2009). Using full genome

data, Maan *et al.* (2011) identified a new serotype of BTV. Rao *et al.* (2013) described typing of two isolates of BTV based on full genome sequence data generated. Full genome data has also been used to determine the origin of the virus strain of BTV responsible for an outbreak in the eastern Netherlands (Maan *et al.*, 2010). It is evident from these studies that comparative genomics has become the most effective way of characterising the viral genomes and studying the differences between viruses.

1.15 AIMS OF THIS INVESTIGATION

To make molecular diagnostics accurate and the vaccine viruses relevant and efficient in eliciting a strong and specific immune response we have to know what is likely to change in the virus proteins, especially VP2. Based on literature our suspicion is that VP2, which carries the neutralising epitopes, does change. We however, do not know how much it varies in AHSV and if the changes will cause antigenic variation. This can be studied by looking at the genomes of historic strains isolated between the years 1961 and 1975 and the recent field isolates (1996 - 2009). We aimed to compare these at both nucleotide sequence and amino acid sequence level, focusing on VP2, VP5 and NS3 of multiple isolates from one serotype.

The objective of this study was therefore to determine the complete genome sequences of numerous historical and recent AHSV isolates, which entail:

- Purifying dsRNA of recent AHSV isolates, encompassing all nine serotypes, and synthesis of cDNA copies of the genome segments.
- Performing ultra-deep sequencing, determining the consensus sequence of each genome segment.
- Performing inter- and intra-serotype pairwise and multiple alignments of the nucleotide and the deduced amino acid sequence.
- Analysis of the predicted antigenicity and hydrophobicity profiles and entropy plots for VP2, VP5 and NS3 proteins of the recent and historic isolates.
- Analysis of the predicted secondary structures for the VP2, VP5 and NS3 proteins of the recent and field isolates.

Chapter 2: Full genome sequencing of recent field isolates of African horse sickness virus

2.1 INTRODUCTION

African horse sickness virus (AHSV) is a member of the *Orbivirus* genus in the family *Reoviridae*, with the genome consisting of ten segments (S1-10) of double-stranded (ds) RNA (Oellerman *et al.*, 1970; Bremer, 1976). The viral genome is enclosed within a double-layered capsid. The outer capsid is composed of the two major structural proteins, VP2 and VP5, with the inner capsid (core) composed of the two major, VP3 and VP7, and three minor, VP1, VP4 and VP6, structural proteins.

Current viral genetics studies make use of full genome sequence data. This was made possible by technological advances, which has progressed from the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) to a massively parallel NGS approach. The latter method simplifies sequencing of full genomes of multiple individual organisms. Using NGS technologies, a large volume of sequence data is produced, with the low number of sequencing runs performed, at a relatively lower cost (Mardis, 2008; Reis-Filho, 2009). Additionally, NGS provides in depth genome sequence data, allowing the identification of variations among individuals of same species (Reis-Filho, 2009).

The aim of this study was to generate complete genome sequences of the nine recent field isolates of AHSV representing serotypes 1 to 9. Prior to this study, ultra-deep sequencing of the selected viral strains had not be completed, thereby hampering genome characterisation of the recent field isolates and comparative genome analysis of the recent strains to the historical reference strains. Whole genome sequence data was generated for each of the recent virus isolates (serotypes 1 to 9), using the Illumina Miseq sequencing platform.

2.2 MATERIALS AND METHODS

2.2.1 Cells and viruses

Baby hamster kidney cell, clone 21 cells (BHK-21), were grown in Dulbecco's modified essential medium (DMEM) containing 4.5 g/L glucose (Lonza, Whitehead Scientific), supplemented with 5% (v/v) fetal bovine serum (FBS), 5 ml of L-glutamine (200 mM) and 5 ml of an antibiotics-antimycotic solution (penicillin-streptomycin-amphotericin B mixture) (Lonza, Whitehead Scientific) in 75 cm^2 cell culture flasks at a temperature of 37°C in an incubator with a 5% CO_2 influx for two or more days until the cells were confluent.

2.2.2 AHSV propagation and RNA extraction

The information of the recent strains of the viruses used in this study and their passage history is listed in Table 2.1. Despite the history of passage in Vero cells, all viruses were cultivated in BHK-21 cells because of the faster growth rate of the cells. This resulted in a high yield of virus RNA. For each infection, two 75 cm^2 flasks containing confluent BHK-21 monolayers, were washed with 5 ml of Hank's balanced salt solution (HBSS) (Lonza, Whitehead Scientific). The monolayers were infected in 5 ml of the DMEM (Lonza, Whitehead Scientific) medium, containing L-glutamine and an antibiotics–antimycotic solution (penicillin-streptomycin-amphotericin B mixture) (Lonza, Whitehead Scientific), in the absence of the serum. The infected cells were incubated for 1 h at 37°C with 5% CO_2 influx prior to the addition of media to a final volume of 15 - 20 ml. This was to allow for virus-cell absorption by decreasing the initial volume of medium. Incubation was then continued at 37°C for 96 h post-infection, after which dsRNA was either extracted from the infected cells or following short-term storage at 4°C.

The AHSV-infected cells showing CPE greater than 80% were scraped from the surface of the flask and collected by centrifugal spinning at 2000 x g for 5 min. TriReagent (1.8 ml) (Sigma-Aldrich) was added to the infected cell pellets. The cell lysates were placed at room temperature for 5 min. Chloroform ($CHCl_3$) was added and the solution incubated at room temperature for 5 min prior to centrifugation at 12000 × g for 40 min, at 4°C. Three phases (aqueous, interphase and organic phase) were formed and the top

aqueous phase containing the RNA was collected. An equal volume of the isopropanol was added, followed by mixing and centrifugation at 12000 x g for 45 min at 4ºC. The supernatant was removed and the precipitate was briefly air-dried and resuspended in nuclease-free water. Lithium Chloride (8 M) (Sigma-Aldrich) was added to a final concentration of 2 M and the mixture was placed overnight at 4ºC. This was followed by centrifugation at 16 000 x g for 40 min, at 4ºC. The supernatant was then transferred from each tube to a clean 1.5 ml microcentrifuge tube. The dsRNA was then purified from the supernatant by absorbtion on the column matrix of the Minelute gel extraction kit (Qiagen), following by washing with a wash buffer and elution.

Table 2.1: Recent field isolates of AHSV sequenced in this study.

Virus	Serotype	Passage History	No. of sequences generated (bp)	Whole genome read count
*HS 107/09	1	#Eq Spln, 2 Vero	228 588	181859
HS 20/09	2	Eq Spln,3 Vero	868 736	447130
HS 109/08	3	Eq Spln,1 Vero	730 262	394891
HS 68/09	4	Eq Lngs, 2 Vero	927 958	500273
HS 35/09	5	Eq Bld, 3 Vero	517 646	287379
HS 63/09	6	Eq Bld, 3 Vero	799 506	452531
HS 89/09	7	Eq Spln, 2 Vero	696 932	398058
HS 29/00	8	Eq Spln, 4BHK	885 712	490136
HS 38/09	9	Eq Spln ,2 Vero	798 252	441155

*Designation of virus isolate i.e. HS107/09: horse sickness isolate number 107, collected in 2009.
#Eq Spln-Equine Spleen, Eq Lngs-Equine lungs, Eq Bld-Equine blood.

2.2.3 Agarose gel electrophoresis

The purified dsRNA was analysed by agarose gel electrophoresis. This was achieved by horizontal casting of the 1% (w/v) agarose gels followed by electrophoresis at 90 V for 45 min, prior to visualisation. Gels were prepared by melting of SeaPlaque® agarose (Whitehead Scientific) in 1 x TAE (40 mM Tris , 20 mM acetic acid, 1 mM EDTA, pH 7.6)

or 1 x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 7.6) and supplemented with 0.5 µg/ml of ethidium bromide (EtBr), so as to allow visualisation of the purified RNA using a G:Box (Syngene) gel imaging system, with an build-in UV transilluminator.

2.2.4 Oligo-ligation

Ligation of the PC3-T7 loop primer to the purified dsRNA was completed essentially as described by Potgieter *et al.* (2009). In short, an anchor primer, PC3-T7 loop (Tib Molbiol.), was ligated to dsRNA (0.5 - 200 ng) in a reaction containing 1 M of HEPES/NaOH, pH 8.0 (Sigma), 1 M of $MgCl_2$ (Sigma), 0.01% of BSA (Takara), 1 mM of ATP, 3 mM of DTT (Roche), 10% of DMSO (Sigma), 20% of polyethyleneglycol (PEG) 6000 (Sigma), the T4 RNA ligase with BSA 0.1 % (Takara) at 37°C for 16 hours. Ligated dsRNA was purified using MinElute gel extraction columns (Qiagen) as per manufacturer's recommendations.

2.2.5 Sequence-independent cDNA synthesis and amplification

Sequence-independent cDNA synthesis and amplification were completed as described by Potgieter *et al.* (2009) with some modifications. The first incubation step for cDNA synthesis was completed at 42ºC for 55 min and not for 45 min. The final step in the synthesis of cDNA was completed at 72°C for 1.5 min and not for 1 min. The initial denaturation step during a cDNA amplification thermocycling profiles was performed for 1.5 min at 98°C and not for 2 min. The denaturation and annealing steps during the 20 cycles of PCR amplification were performed at 94°C for 15 sec and 65°C for 30 sec, and not 94°C for 30 sec and 67°C for 30 sec, respectively. Following PCR amplification, amplicons were then analysed by agarose gel electrophoresis (Section 2.2.3) in a 1.2 % (w/v) agarose gel.

2.2.6 cDNA library construction and sequencing

At least 70 ng of cDNA copies of the AHSV dsRNA genome segments of each virus were submitted to the ARC-OVI Biotechnology platform in South Africa for paired-end library

preparation and sequencing. Libraries were prepared by making use of the Nextera DNA library preparation kit (Illumina), as per manufacturer's guidelines. Paired-end, 250-bp multiplex sequencing was completed on the Illumina MiSeq desktop sequencer.

2.2.7 Genome assembly

The forward and reverse reads of the NGS data were imported as paired-ends with the distance of 250 bp into the CLC-Bio Genomic Workbench (version 6.0) software (CLC Bio, Denmark). The paired-end sequencing reads were trimmed at both the 5' and 3' end, to remove the adapter sequences. *De novo* assembly was carried out for all the cDNA copies of the genome of each AHSV isolate. All parameters were set at default settings except for the minimum contig length, which was set to 500 bp to enable assembly of the smallest genome segment (758 bp) (Roy *et al.*, 1994). The identity of each contig obtained, following *de novo* assembly, was confirmed by homology search using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Genome reference assembly was completed for each of the recent isolates using the sequencing reads of the genomes' cDNA copies and sequence data for other AHSV strains from GenBank, as reference templates. All parameters were set as default. The resulting assembled genome sequences were aligned to those generated by *de novo* assembly using the CLC-Bio Genomic Workbench (version 6.0) software. Resulting conflicts were resolved by the software, by identifying variants in the respective assemblies (*de novo* and map to reference assembly) and the coverage of each. The variants showing the highest coverage in the individual assemblies were included in the final consensus sequence for each of the respective genome segments.

2.2.8 AHSV field isolates sequence analysis and characterisation

Multiple sequence alignment of the AHSV serotype 1 to 9 nucleotide and amino acid sequences of all ten genome segments, of the recent field isolate were obtained. All alignments were completed using default alignment parameters within the CLC analysis software. Using the sequence alignments, the 5' and 3' ends and the middle region of the

individual consensus sequences were analysed and compared for all genome segments of all nine recent field isolates of AHSV serotypes 1 to 9. The consensus sequences of the individual genome segments of AHSV serotypes 1 to 9 were analysed for all nine isolates. For the latter analysis open reading frames were identified, sequence length of individual genome segment were analysed.

2.3 RESULTS

2.3.1 Preparation and characterisation of purified dsRNA

The first step towards the complete genome characterisation of several recent field isolates of AHSV, encompassing all nine serotypes of the virus, was to propagate the virus isolates in order to extract their dsRNA genomes. Following infection of BHK cells with each of the selected isolates, dsRNA was purified and analysed by agarose gel electrophoresis. There were no visible ssRNA carried over after the differential LiCl precipitation steps (Fig. 2.1). For each serotype, eight dsRNA bands were observed. This results are in agreement with results reported by Bremer (1976). The first six bands from the top comprises of single genome segments, while the 7th band contains segments 7, 8 and 9 (Bremer, 1976) and, therefore, appears more intense. The fastest migrating band is segment 10, and because of the relatively low concentration in the samples, it appears less intense.

2.3.2 Preparation and analysis of AHSV genomic cDNA

In order to generate complete genome sequences of the selected viruses, cDNA copies of the viral genomes were synthesised. The purified dsRNA samples of each of the selected viral strains were used as templates for sequence-independent cDNA synthesis and amplification. The final cDNA products were analysed by agarose gel electrophoresis prior to being subjected to ultra-deep sequencing. Amplification of the cDNA copies of the individual genome segments was successful across all serotypes. AHSV serotypes 1, 2,

7 and 9 however showed a low concentration of S1 and S2 (Fig. 2.2). This results are in agreement to that reported by Potgieter *et al.* (2009).

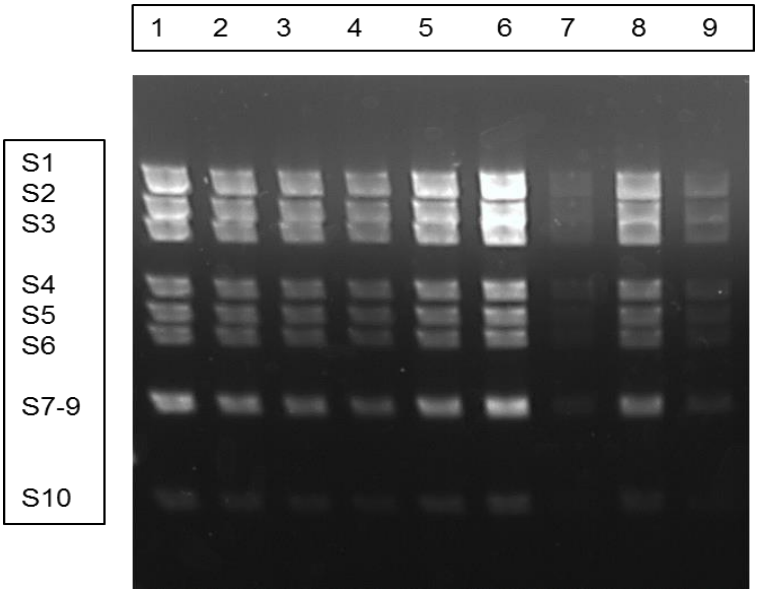

Figure 2.1: Agarose gel electrophoresis of the dsRNA genome segments (S1-10) purified from recent AHSV field isolates, each representing one of the nine serotypes. The lane numbers correlate with the specific serotype (AHSV 1 to 9). The different genome segments or genome segments that co-migrate are indicated to the left of the figure.

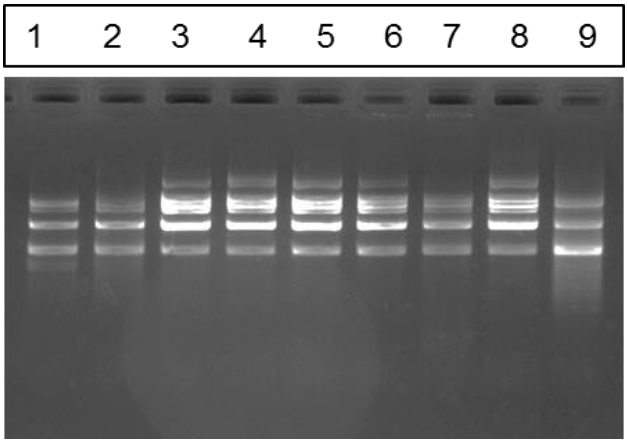

Figure 2.2: Agarose gel electrophoresis of the cDNA of each of the ten dsRNA genome segments (S1-10) of each of the recent AHSV field isolates. The lane numbers correlate with the specific serotype (AHSV 1 to 9).

2.3.4 Sequence determination of the recent AHSV isolates

In order to characterise the genomes of each of the selected recent viral strains, encompassing all nine serotypes of AHSV, complete genome sequencing was completed. The generated cDNA was used in fragmented library preparation and subjected to MiSeq sequencing. The number of reads generated per genome ranged from 228 588 - 500 273 including both single reads and paired reads (Table 2.1). AHSV genome segments with the highest number of total reads and the highest coverage were segment 10 encoding NS3, segment 9 encoding NS2 and segment 5 encoding NS1 (Table 2.2). For all the selected viral genomes sequenced, the number of reads per genome segment showed to be in inverse proportion to the sizes of the segments, with smaller segments showing a high read count.

2.3.5 AHSV field isolates sequence assembly and characterisation

Consensus sequences of the assembled genome segments of the nine recent field isolates representing serotypes 1 to 9 were submitted to GenBank and accession numbers were assigned as summarised in Table 2.3. The total genome size of each AHSV isolate was determined to be ca.19510 bp, with genome segments sizes ranging from 758 to 3965 bp in length. Analysis of sequencing data indicates that the non-coding regions of the genome segments share a number of conserved nucleotide bases at the 5’ and 3’ termini (Table 2.4). In accordance with the consensus AHSV terminal sequences, 5’ and 3’ terminal sequences of all isolates demonstrated the 5’ - GUUUA and CAUAC - 3’, respectively (Roy *et al.*, 1994, 2006). Partial complementarity of nucleotide sequences at the 5’ and 3’ termini were observed for each genome segment. This result is in agreement with that reported by Roy *et al.* (1994, 2006). All of the genome segments with the exception of segment 9 and 10 had a single open reading frame (ORF). For genome segment 10, ORFs encoding two polypeptides, previously assigned as NS3 and NS3A were observed as reported by van Niekerk *et al.* (2001). In all of the AHSV isolates characterised in this study, genome segment 9, encoding VP6, contained an overlapping ORF, encoding NS4 type I or II protein. The ORF of NS4 type I or II protein,

overlapping the VP6 ORF, was identified by the starting codon of AUG at positions 12 - 218 and stop codons UGA/UAA at positions 573 - 3930 respectively (Zwart *et al.*, 2015).

Table 2.2: Average coverage of the individual genome segments of all nine serotypes of the selected recent field isolates

AHSV isolate	AHSV Serotype	VP1	VP2	VP3	VP4	NS1	VP5	VP6	VP7	NS2	NS3
HS 107/09	**1**	*134.86	269.40	131.53	1004.96	5606.15	3176.72	1393.51	4003.56	6208.32	13461.36
HS 20/09	**2**	600.78	601.27	840.28	2467.80	5990.08	201.90	5849.25	11434.10	20383.79	22217.62
HS 109/08	**3**	170.54	103.70	1223.86	4615.26	8804.25	7540.84	6348.21	9245.99	15180.35	7779.16
HS 68/09	**4**	293.46	1588.94	1932.66	6555.93	12687.10	3535.98	5016.62	12188.77	14612.11	11913.74
HS 35/09	**5**	141.86	423.62	1459.26	4198.86	7654.68	4797.82	2892.84	6949.54	8778.90	6600.94
HS 63/09	**6**	128.59	552.74	1269.22	5474.68	10101.95	3776.04	5233.05	12983.13	15176.80	11636.51
HS 89/09	**7**	189.09	214.59	459.52	3190.81	7103.68	4002.65	2169.45	12639.74	14258.07	24562.39
HS 29/00	**8**	272.24	842.48	1913.05	5693.43	10884.82	1445.66	7684.26	11182.14	15168.58	12054.51
HS 38/09	**9**	876.33	1209.54	1111.08	3383.85	10045.76	2408.24	4018.30	5505.92	13211.91	39635.90

*Using the CLC genomic workbench software, the average coverage of each genome segment was determined by counting together all the bases of the aligned sequences (during reference assembly) and dividing that by the length of the used reference genome segment sequences from GenBank.

Table 2.3: GenBank accession numbers of the consensus nucleic acid sequences of the individual genome segments of the selected recent AHSV isolates.

	HS107/09	HS 20/09	HS 109/08	HS 68/09	HS 35/09	HS 63/09	HS 89/09	HS 29/00	HS 38/09
VP1	KP939368	KP939419	KP939478	KP939575	KP939702	KP939807	KP939934	KP940001	KP940125
VP2	KP939373	KP939425	KP939487	KP939588	KP939712	KP939820	KP939941	KP940013	KP940137
VP3	KP939378	KP939431	KP939496	KP939601	KP939722	KP939833	KP939948	KP940025	KP940149
VP4	KP939383	KP939437	KP939505	KP939614	KP939732	KP939846	KP939955	KP940037	KP940161
VP5	KP939393	KP939449	KP939523	KP939640	KP939752	KP939872	KP939969	KP940061	KP940185
VP6	KP939408	KP939467	KP939550	KP939679	KP939782	KP939911	KP939990	KP940097	KP940220
VP7	KP939398	KP939455	KP939532	KP939653	KP939762	KP939885	KP939976	KP940073	KP940197
NS1	KP939388	KP939443	KP939515	KP939627	KP939742	KP939859	KP939962	KP940049	KP940173
NS2	KP939403	KP939461	KP939541	KP939666	KP939772	KP939898	KP939983	KP940085	KP940209
NS3	KP939413	KP939473	KP939559	KP939692	KP939792	KP939924	KP939997	KP940109	KP940232

Table 2.4: Characteristics of the assembled cDNA copies of genome segments (S1-10) of recent field isolates of AHSV serotype 1 to 9.

Genome Segment	Size (bp)	Coding region (position ORF)	Virus protein	Length of encoded protein (amino acids)	5' Terminal sequence (positive strand)	3' Terminal sequences (positive strand)
1	3965	13 - 3930	VP1	1305	5' - GTTTAT	ACTTAC - 3'
2	2988 - 3229	12 - 3173/3186/3195	VP2	1051	5' - GTTTA$^{A/T}$	$^{A/C}C^{T/A}$TAC - 3'
3	2792	27 - 2744	VP3	905	5' - GTTTAT	ACTTAC - 3'
4	1975 - 1978	12 - 1940	VP4	642	5' - GTT$^{A/T}$AT	CCTTAC - 3'
5	1747 - 1749	36 - 1681	NS1	548	5' - GTTAAA	ACTTAC - 3'
6	1564 - 1566	20 - 1537	VP5	504	5' - GTTTAT	ACATAC - 3'
7	1167	18 - 1139	VP7	373	5' - GTT$^{T/A}$AA	ACTTAC - 3'
8	1166 - 1170	23 - 1124	NS2	365	5' - GTTTAA	ACATAC - 3'
9	1075 - 1172	193 - 660/18 - 1145	NS4/VP6	125 - 154/370	5' - GTT$^{A/T}$AA	ACTTAC - 3'
10	756 - 764	19/52 - 679	NS3/NS3A	216/217	5' - GTT$^{T/A}$AA	ACTTAC - 3'

2.4 DISCUSSION

Outbreaks of AHS have been recorded in southern Africa after 1652, since the introduction of horses and mules from Europe and the Far East by the settlers. Since 1933, blood and tissue samples are continuously submitted to the OIE reference laboratory for AHS at the Onderstepoort Veterinary Institute (OVI) in South Africa. The original samples and virus isolates are stored in the sample and virus bank at the OVI. The animal, sample and viral passages histories are documented and filed. Although the sample collection to date, including those used in this study, were confirmed to contain the causative agent (AHSV) and the specific serotypes were identified, limited full virus genome sequences have been determined, especially with ultra-deep sequencing. The complete genome sequences of the reference isolates were recently determined by ultra-deep sequencing (Potgieter *et al.,* 2009; Koekemoer, 2013; unpublished data) and are available within GenBank. Previously, some of the cloned cDNA copies of the individual genome segments of certain AHS isolates (mostly reference strains), were sequenced using the Sanger method, but this approach, sequencing of the cloned individual cDNA copies of the genome segments, has limitations and does not allow for the identification of variations within isolates (quasispecies) or between isolates (Domingo *et al.,* 1996; Reis-Filho, 2009). In this study, cDNA copies of the complete segmented dsRNA genome sequences of nine recent field isolates of AHSV serotypes 1 to 9 (2000; 2008 - 2009) were determined by an ultra-deep sequencing approach.

To characterise the genomes of the above mentioned AHSV isolates, virus propagation was completed, followed by sequence-independent cDNA synthesis and ultra-deep sequencing. Genome assembly and sequence analysis were performed with CLC-Bio Genomic Workbench (version 6). Migration patterns of the dsRNA samples through a horizontal agarose gel showed that each virus isolate contained a single genotype of AHSV. By comparison of our data sets with AHSV data in the NCBI database, we were able to identify each segment of each isolate and to confirm that full-length sequences were obtained. In addition, the serotype of each isolate was confirmed by comparative analysis of segment 2 sequences to known AHSV sequence data. Segments 2 encodes VP2, which is the main serotype-specific determinants of AHSV (Burrage *et al.*, 1993),

also the most variable segment. The conserved hexanucleotide consensus sequences at the 5' and 3' termini of viruses in the *Orbivirus* genus as described by Mizukoshi *et al.* (1993) were observed (Table 2.4). Each genome segment showed the partial inverted complimentary between the 5' and 3' termini as previously reported by Roy *et al.* (1994).

The sequencing coverage was sufficient to not only enable full-length genome segment and complete genome characterisation, but also the identification of sequence variants as described in Chapter 3. The average read coverage of each segment correlated with the concentration of cDNA copies of each dsRNA genome segment. Genome segment 1 and 2, yielded low concentration of cDNA for AHSV-1,-2,-7 and -9, thereby resulting in low sequencing coverage for each isolate, by comparison to other segments' cDNA copies. This result is in agreement with finding reported previously, whereby cloning and sequencing of the large segment was found to be difficult yielding a low concentration in cDNA samples (Potgieter *et al.*, 2009).

Chapter 3: Complete genome comparison of recent field and historic isolates of African horse sickness virus.

3.1 INTRODUCTION

A study on the level of variation between the genomes of organisms can provide valuable information for vaccine and diagnostic development. Comparison of the individual genome segments of the African horse sickness virus (AHSV) completed this far has shown variation even between strains of the same serotype (Vreede & Huismans, 1998; Maree & Huismans, 1997; Huismans *et al.*, 2004). Such information has been used successfully in the development of diagnostic assays by targeting conserved regions of the genome (Weyer *et al.*, 2012, Guthrie *et al.*, 2013). The comparison of the whole genomes of viruses isolated over a time span is yet to be completed for AHSV. The aim of this chapter was, therefore, to determine the level of genetic variation between the recent AHSV field isolates and the historic reference strains. Additionally, to complete a comparative analysis of genome segment 2, 6 and 10 of one serotype (AHSV-9) using the historical isolates, the selected recent isolates and the additional recent field isolates. This analysis is particularly important for the development of next generation vaccines and new diagnostic assays for AHS. Knowing the level of variation between the isolates assists in determining the appropriate strains for use in the development of an effective vaccine and in designing accurate molecular diagnostic assays. Also, in terms of the diagnostic assays based on serology (VN and ELISA) pure viral strains are required to raise antibodies that will specifically bind or neutralise the virus that is being targeted.

3.2 MATERIALS AND METHODS:

3.2.1 Comparative sequence analysis

Complete genome sequences of the AHSV serotype 1 to 9 of the nine historic reference strains (1961 - 1975), and the seven additional field isolates (1994 - 2001) of serotype 9, were determined by ultra-deep sequencing and generously provided by Dr. Koekemoer and Mr. Ngoveni, respectively, from the OVI. The GenBank accession numbers of the historic reference strains and the seven additional field isolates are summarised in Table 3.1 and 3.2, respectively. Full consensus genome sequences of the nine recent field isolates (AHSV-1 to -9) (2000 - 2009) were also determined by ultra-deep sequencing (Chapter 2). Using the CLC-Bio Genomics Workbench (Version 6) (CLC Bio, Denmark) (CLC), the ORF of each of ten genome segments of each isolates were translated (*in silico*), using the standard genetic code translation table and the correct ORF selected. Intra- and inter-serotype alignments of the nucleotide and amino acid sequences of the corresponding genome segments were completed for all ten genome segments of the nine recent field isolates and the nine historic strains of AHSV. Multiple sequence alignment of the AHSV-9 nucleotide and amino acid sequences of the three most divergent proteins (VP2, VP5 and NS3), of the seven additional field isolates, the historic reference strain and the recent field isolate were obtained. All alignments were completed using default alignment parameters within the CLC analysis software. Using all nucleotide and amino acid sequences alignments obtained, the percentage sequence identities were determined within the CLC analysis software.

Table 3.1: GenBank accession numbers of the nucleic acid sequences of the individual genome segments of the historic reference strains of AHSV.

	HS 29/62	HS 82/61	HS 13/63	HS 32/62	HS 30/62	HS 2/75	HS 31/62	HS 10/62	HS90/61
VP1	KP939371	KP939423	KP939479	KP939571	KP939701	KP939803	KP939930	KP939998	KP940129
VP2	KP939376	KP939429	KP939488	KP939584	KP939711	KP939816	KP939937	KP940010	KP940141
VP3	KP939381	KP939435	KP939497	KP939597	KP939721	KP939829	KP939944	KP940022	KP940153
VP4	KP939386	KP939441	KP939506	KP939610	KP939731	KP939842	KP939951	KP940034	KP940165
VP5	KP939396	KP939453	KP939524	KP939636	KP939751	KP939868	KP939965	KP940058	KP940189
VP6	KP939411	KP939471	KP939551	KP939675	KP939781	KP939907	KP939986	KP940094	KP940224
VP7	KP939401	KP939459	KP939533	KP939649	KP939761	KP939881	KP940070	KP940070	KP940201
NS1	KP939391	KP939447	KP939515	KP939623	KP939741	KP939855	KP939958	KP940046	KP940177
NS2	KP939406	KP939465	KP939542	KP939662	KP939771	KP939894	KP939979	KP939979	KP940213
NS3	KP939416	KP939477	KP939560	KP939688	KP939791	KP939920	KP939993	KP940106	KP940236

Table 3.2: GenBank accession numbers of the nucleic acid sequences of S2 (VP2), S6 (VP5) and S10 (NS3) of the additional recent isolates of AHSV.

	HS 49/02	HS 2/03	HS 15/02	HS 22/03	HS 43/01	HS6/01
VP2	KP940139	KP940135	KP940130	KP940133	KP940138	KP940140
VP5	KP940187	KP940183	KP940178	KP940181	KP940186	KP940188
NS3	KP940234	KP940230	KP940225	KP940228	KP940233	KP940235

An estimation of the antigenic regions within the VP2 and VP5 proteins of AHSV-9 was completed using the Kyte-Doolittle (Kyte & Doolittle, 1982) algorithm, for the seven additional isolates, the recent field isolate and the historic strain. The hydrophobicity of the NS3 protein sequences of the AHSV-9 of the seven additional isolates, the recent field isolate and the historic strain were also estimated using the welling (Welling *et al.*, 1985) algorithm. The window sizes were set to 11 for both predictions within the CLC analysis software. The aligned deduced amino acid sequences of S2 (VP2) and S6 (VP5) genes of AHSV-9 of the seven additional field isolates, the recent field and historic reference strain, were analysed for regions showing most variation between the isolates by determining site-specific entropy throughout sequence length using the Data Analysis in Molecular Biology and Evolution (DAMBE) algorithm (Xia, 2013). Using a hidden Makov model (HMM), the secondary protein structures were predicted within the CLC analysis software, for the most divergent proteins (VP2, VP5 and NS3) using the deduced amino acid sequences of the seven additional field isolates, the recent field and the historic reference strain.

3.2.2 Detection of quasispecies variants at genome segment level for the more recent field isolates

Within the CLC-Bio Genomic Workbench (version 6.0) software (CLC Bio, Denmark), paired-end reads of the cDNA copies of the genomes of the recent field isolates AHSV 1 to 9, were combined and 20 nucleotide bases inclusive of the adapter sequences trimmed from each end. The trimmed sequence data of the recent field isolates was subsequently mapped to the previously determined consensus nucleotide sequence (Chapter 2) for each genome segment using default parameter settings within the CLC analysis software. Output options were selected to create reads tracks. Probabilistic variant detection was carried out using with the following parameters: “read filter” to ignore non-specific matches and broken pairs, “minimum coverage” of 10, “variant probability” of 0.90 and “variant filters” set to require presence in both forward and reverse reads. The “ploidy” parameter was set to a maximum of 4 variants and the genetic code was set to standard.

3.3 RESULTS:

3.3.1. Comparative genomic analysis of the recent field isolates of the AHSV serotypes 1 to 9

Inter-serotype comparison of the nine recent field isolates (2000 - 2009) of AHSV serotypes 1 to 9 sequenced in Chapter 2, were completed for all ten genome segments, in order to determine the level of variation for the corresponding genome segments. The analysis were completed by multiple sequence alignments of the nucleotide and deduced amino acid sequences of the corresponding genome segments. Pairwise sequence similarities are summarised in Table 3.3. S2 (VP2) is the most variable, followed by S10 (NS3) and S6 (VP5). The multiple sequence alignments of the VP2, VP5 and NS3 sequences of different serotypes showed variation between 28 - 52%, 1 - 24% and 1 - 35%, respectively. These results show agreement to that reported by Huismans *et al.* (2004). Comparison of the VP3 and VP7 sequences of the AHSV, serotypes 1 to 9, indicated that 99.6% and 99.7% amino acids were identical in type and position (Table 3.3) respectively, confirming the conservative nature of these proteins within the serogroup. Comparison of the VP1, VP4, NS1-2 and VP6 sequence of AHSV serogroup also indicated that they are highly conserved with lowest sequence identity of 91.87% observed for VP6 (Table 3.3).

3.3.2. Comparative genomic analysis of the historic reference strains and recent field isolates of AHSV serotypes 1 to 9

Intra-serotype comparison of the nine recent field isolates (2000 - 2009) sequenced in the previous chapter and the nine historic reference strains (1961 - 1975) (Table 3.1) of AHSV serotypes 1-9, were completed. This was achieved by using the nucleotide and amino acid sequences of the ten genome segments so as to determine the level of genetic variation of the corresponding genes between the years. Pairwise alignments of the nucleotide and the deduced amino acid sequences of the individual genome segments of the recent and historic strains showed most variation for serotype 9 and the least for serotype 1 with maximum variability of 28% and 8% on nucleotide and amino acid level, respectively, observed for comparison of the corresponding segments of the reference

and recent strains of AHSV. The percentage sequence identities for intra-serotype comparison of the individual genome segments are summarised in Table 3.3.

3.3.3 Further analysis of the VP2, VP5 and NS3 proteins of the AHSV-9 for the seven recent isolates and the reference strain.

The number and location of sequence variations that have accumulated between the years, 1961 and 2009, within a single serotype were determined by further analysis of genome segments 2 (VP2), 6 (VP5) and 10 (NS3). In the latter comparative analysis, the consensus sequences of the recent AHSV-9 field isolate (HS 38/09) determined in this study (Section 2.3.4), as well as those of an additional six recent AHSV-9 field isolates (Table 3.2) were compared to that of the AHSV-9 reference strain (HS90/61). S2 (VP2), S6 (VP5) and S10 (NS3) encode the most variable proteins for AHSV and showed most variation for inter-serotype comparison of the recent field isolates (Section 3.3.2). Intra-serotype comparison of the recent isolates and the reference strains showed the most variation within AHSV-9 isolates. As a result, it was of interest to determine variation over time focusing on VP2, VP5 and NS3 of AHSV-9. From the alignments of the nucleotide and deduced amino acid sequences (Appendix A1 - A3), the percentage sequence identities were determined as shown in Table 3.4 to 3.6.

A number of amino acid substitutions were observed within the VP2, VP5 and NS3 proteins of the recent AHSV-9 isolates by comparison to those of the historic strain of AHSV-9 (HS 90/61), as shown in Appendix B1 - B3. The significance of these changes were evaluated by examination of the entropy, antigenicity and hydrophobicity plots and the predicted secondary structures of the deduced amino acid sequences. Regions with putative important amino acid changes, as evidenced by changes in the predicted antigenicity, hydrophobicity plots and protein secondary structures between the recent field isolates and reference isolates, were identified (Fig. 3.1 - Fig. 3.3).

3.3.3.1 VP2

By comparison to the reference AHSV-9 isolate, three primary regions within the VP2s of the six recent AHSV-9 isolates contained non-synonymous substitutions associated with

altered predicted secondary structures and antigenicity profiles (Fig. 3.1). These regions included amino acid residues 100 - 300, 551 - 601 and 851 - 951. The entropy scores at these regions were also high. Of the 27 amino acid changes observed for these regions, 13 were conservative, eight were semi-conservative and six were non-conservative (Table 3.7). Eight of the non-conservative amino acid changes were within and adjacent to the previously identified epitope regions (200 - 400 and 550 - 680) of the VP2 protein (Martínez-Torrecuadrada & Casal, 1995; Bentley *et al*., 2000; Martínez-Torrecuadrada *et al*., 2001).

3.3.3.2 VP5

Sixteen non-synonymous substitutions were observed within the VP5 proteins of the seven recent AHSV-9 isolates against the AHSV-9 reference strain (Appendix B2). Twelve of the substitutions were conservative, three were semi-conservative and one was non-conservative. The substitutions corresponded with changes in the antigenicity profiles of the VP5 proteins of the recent isolates in comparison to that of the reference strain (Fig. 3.2). High entropy values were also observed for the regions containing the amino acid changes. VP5 proteins of all isolates showed the most variation for amino acid residues 100 - 201 as visible from the antigenicity and entropy plots (Fig. 3.2). There were no differences observed for the predicted secondary structures of the VP5 proteins of the recent isolates in comparison to that of the reference strain.

3.3.3.3 NS3

The conserved regions of the NS3 protein, the NS3A initiation codon, a proline-rich region, a conserved region from amino acids residues 43 to 92 and the two hydrophobic regions (HD-1 and -2) are evident for the NS3s of the recent and reference strains (van Niekerk *et al.*, 2001). Twenty-one non-synonymous substitutions were observed within the NS3 proteins of the recent AHSV-9 isolates in comparison to the AHSV-9 reference strain. Conservative, semi-conservative and non-conservative amino acid substitutions were contained within the HD1, the C- and N-terminal ends and the variable region between HD-1 and HD-2 of the NS3 sequences of the seven AHSV-9 recent isolates in

comparison to the AHSV-9 reference strain NS3 sequence (Appendix B3). The amino acid changes within the seven AHSV-9 NS3 sequences in comparison to the reference strain AHSV-9 NS3 sequence, correlated with the differences in the hydrophobicity profiles of the NS3 sequences (Fig. 3.3). The predicted secondary protein structures of the NS3 sequences of the seven recent and reference strains were identical.

Table 3.3: Summary of percentage sequence identity between the ten corresponding genome segments (S1-S10) of the historical reference and recent field isolates encompassing all nine serotypes of AHSV.

		Comparative nucleotide sequence (nt) analysis		Comparative amino acid sequence (aa) analysis	
Genome segment (No. of bp)	Encoded protein (No. of aa)	Inter –serotype (field)	Intra –serotype (field vs ref)	Inter-serotype (field)	Intra –serotype (field vs ref)
S1 (3965)	VP1 (1305)	88.73% - 99.70%	88.95% - 99.95%	98.16% - 99.77%	98.08% - 99.85%
S2 (3217 - 3229)	VP2 (1051 - 1060)	54.80% - 68.09%	89.98% - 99. 88%	47.89% - 71.50%	95% - 99.81%
S3 (2792)	VP3 (905)	95.16% - 99.61%	94.05% - 99.86%	99.56% - 100%	99.45% - 100%
S4 (1978)	VP4 (642)	93.48% - 99.90%	88.98% - 99.95%	97.51% - 99.84%	94. 71% - 100%
S5 (1747 - 1749)	NS1 (548)	95.88% - 99.71%	94.79% - 98.63%	97.63% - 99.64 %	98.17% - 99.45 %
S6 (1566)	VP5 (505)	71.3% - 99.5%	81.48% - 99.87%	75.84% - 99.01%	95. 25% - 100%
S7 (1167)	VP7 (349)	94.77% - 99.83%	88.35% - 99.91%	99.73% - 100%	98.29% - 100%
S8 (1154 - 1166)	NS2 (365)	95.63% - 99.91%	95.63% - 100%	98.91% - 100%	98.36% - 100%
S9 (1160 - 1172)	VP6 (366 –370), NS4 (193 - 660)	71.95% - 100%	72.11% - 99.83%	91.87% - 100%	95.25% - 100%
S10 (756 - 763)	NS3 (217 - 218), NS3A (207 - 208)	66.45% - 99.47%	87. 43% - 99.61%	65.16% - 99.09%	91.47% - 99.09%

ref: reference isolates; field: more recent field isolates; vs: versus

Table 3.4: Percentage sequence-identity matrix of the AHSV-9 segment 2 and deduced VP2 aligned sequences of the six recent isolates and the reference strain.

	HS 90/61	HS 43/01	HS 15/02	HS 2/03	HS 49/02	HS 22/03	HS 38/09
HS 90/61	100	95.47*	95.38	95.28	95.28	95.28	95.19
HS 43/01	90.04#	100	99.91	99.81	99.81	99.81	99.72
HS 15/02	90.01	99.72	100	99.72	99.72	99.72	99.62
HS 2/03	90.07	99.66	99.69	100	99.62	100	99.53
HS 49/02	90.01	99.72	99.81	99.69	100	99.62	99.53
HS 22/03	90.01	99.72	99.81	99.69	100	100	99.53
HS 38/09	89.98	99.56	99.53	99.47	99.53	99.53	100

*Percentage identities (upper right) for the comparison of the amino acids sequences
#Percentage identities (lower left) for the comparison of the nucleotide sequences

Table 3.5: Percentage sequence-identity matrix of the AHSV-9 segment 6 and deduced VP5 aligned sequences of the seven recent isolates and the reference strain.

	HS 90/61	HS 2/03	HS 6/01	HS 43/01	HS 15/02	HS 49/02	HS 22/03	HS 38/09
HS 90/61	100	98.46*	98.46	98.46	98.08	98.66	98.66	98.08
HS 2/03	93.1#	100	100	100	99.62	99.42	99.42	98.08
HS 6/01	93.1	100	100	100	99.62	99.42	99.42	98.08
HS 43/01	93.04	99.81	99.81	100	99.62	99.42	99.42	98.08
HS 15/02	93.1	99.68	99.68	99.49	100	99.04	99.04	97.7
HS 49/02	93.23	99.81	99.81	99.62	99.87	100	100	97.89
HS 22/03	93.1	99.87	99.87	99.68	99.55	99.68	100	97.89
HS 38/09	93.61	95.91	95.91	95.72	95.79	95.91	95.79	100

*Percentage identities (upper right) for the comparison of the amino acids sequences
#Percentage identities (lower left) for the comparison of the nucleotide sequences

Table 3.6: Percentage sequence-identity matrix of the AHSV-9 segment 10 and deduced NS3 aligned sequences of the seven recent isolates and the reference strain.

	HS 90/61	HS 2/03	HS 6/01	38/09	HS 15/02	HS 43/01	HS 49/02	HS 22/03
HS 90/61	100	99.54*	99.08	99.08	97.7	92.63	93.09	90.78
HS 2/03	86.9#	100	99.54	99.54	98.16	92.63	93.55	90.78
HS 6/01	86.77	99.6	100	99.08	97.7	93.09	94.01	90.78
HS 38/09	86.64	99.47	99.6	100	98.16	92.63	94.01	90.78
HS 15/02	87.04	99.6	99.74	99.6	100	91.71	92.63	90.78
HS 43/01	87.17	97.09	96.96	96.83	96.96	100	97.24	94.01
HS 49/02	93.39	88.62	88.49	88.36	88.76	88.89	100	94.47
HS 22/03	93.39	88.62	88.49	88.36	88.76	88.89	100	100

*Percentage identities (upper right) for the comparison of the amino acids sequences
#Percentage identities (lower left) for the comparison of the nucleotide sequences

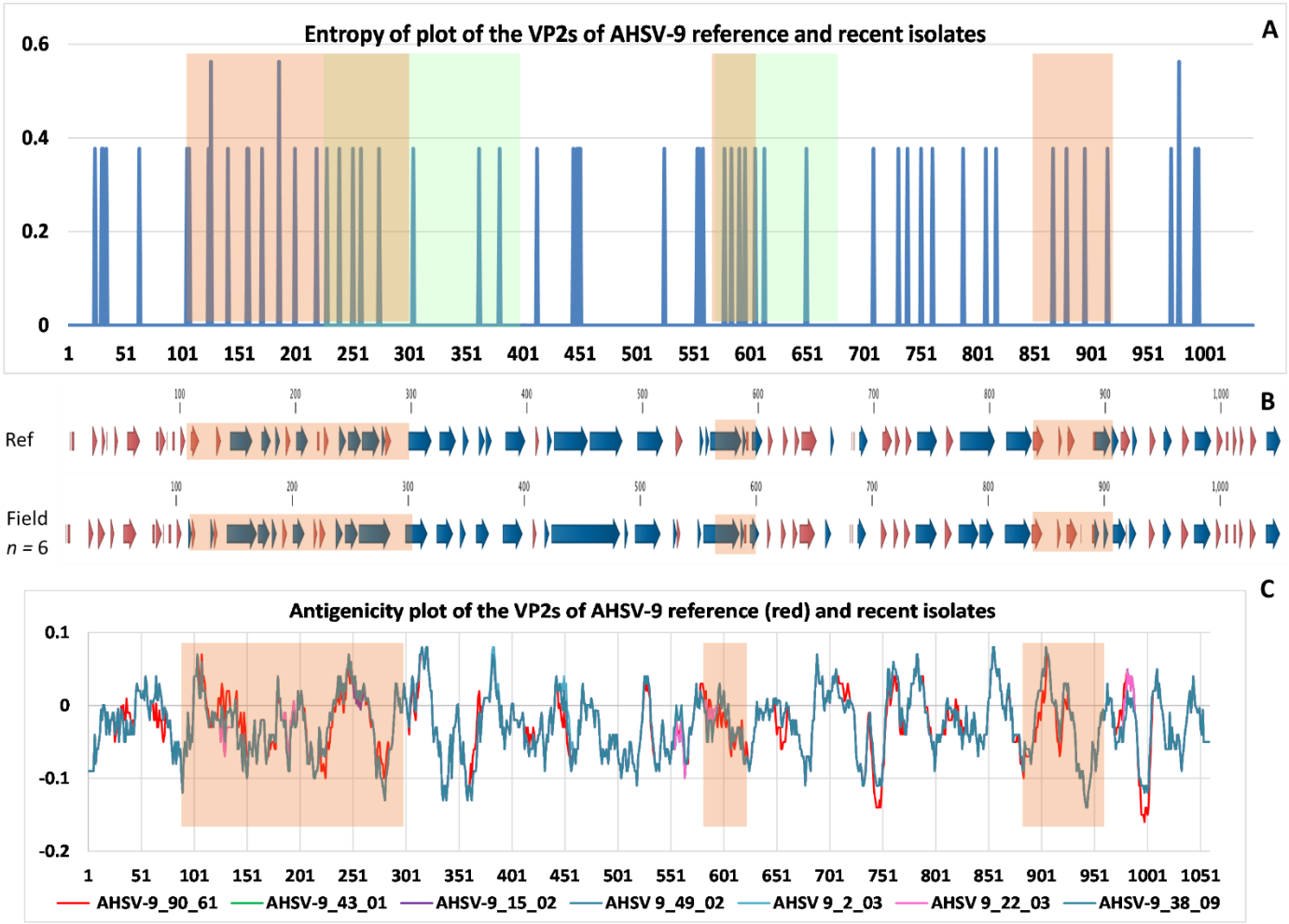

Figure 3.1: Entropy (A), antigenicity (C) and secondary structure (B) predictions of VP2 proteins of AHSV-9 historic and recent strains. Known epitope-containing regions (200 - 400 and 550 - 680) (Bentley *et al.*, 2000; Martínez-Torrecuadrada & Casal, 1995; Martínez-Torrecuadrada *et al.*, 2001) are highlighted in blue (A). Regions containing non-synonymous amino acid substitutions in correlation with the changes in the secondary structure and/or antigenicity properties of the recent strains in comparison to those of the reference strain are highlighted in orange. The blue and red arrows (B) indicate the alpha-helices and beta-sheets, respectively.

Table 3.7: Non-synonymous amino acid substitutions in three regions of the VP2s of seven recent AHSV-9 isolates, by comparative analysis to the VP2 of the AHSV-9 reference strain.

Region 1 Amino acid 100 - 300	Regions 2 Amino acid 551 - 601	Regions 3 Amino acid 851 - 951
#100 Thr → Ile	559 Glu → Lys	872 Phe → Ser
112 Phe → Ser	561 Arg → Gln*	884 Ser → Leu
129 His → Tyr*	564 Thr → Ser	900 Ile → Ala
131 Thr → Ile	583 Asp → Asn*	920 Ile → Val
146 Thr → Met	589 Lys → Arg	
163 Gly → Ser	596 Arg → Lys	
164 Ser → Gly	601 Glu → Asp	
176 Gly →Glu*		
191 Thr →Ala		
205 Ile → Met		
224 Gly → Asp*		
233 Gly → Glu*		
244 Arg → Lys		
256 Ala → Val		
263 Asn → Ser		
279 Val → Ile		

#The numerical value is the amino acid residue in VP2. The first amino acid refers to the residue in the AHSV-9 reference strain (HS 90/61) while the second amino acid refers to the substitution in the six recent AHSV-9 isolates (HS 43/01, HS 15/02, HS 2/03, HS 49/02, HS 22/03 and HS 38/09). The asterisks indicate non-conservative mutations. Except for 131 Thr→Ile and 191 Thr → Ala only in HS 49/02 and HS 22/03, 256 Ala →Val only in HS 15/02, 559 Glu → Lys and 589 Lys → Arg only in HS 38/09 the remaining substitutions listed occurred in all six recent isolates

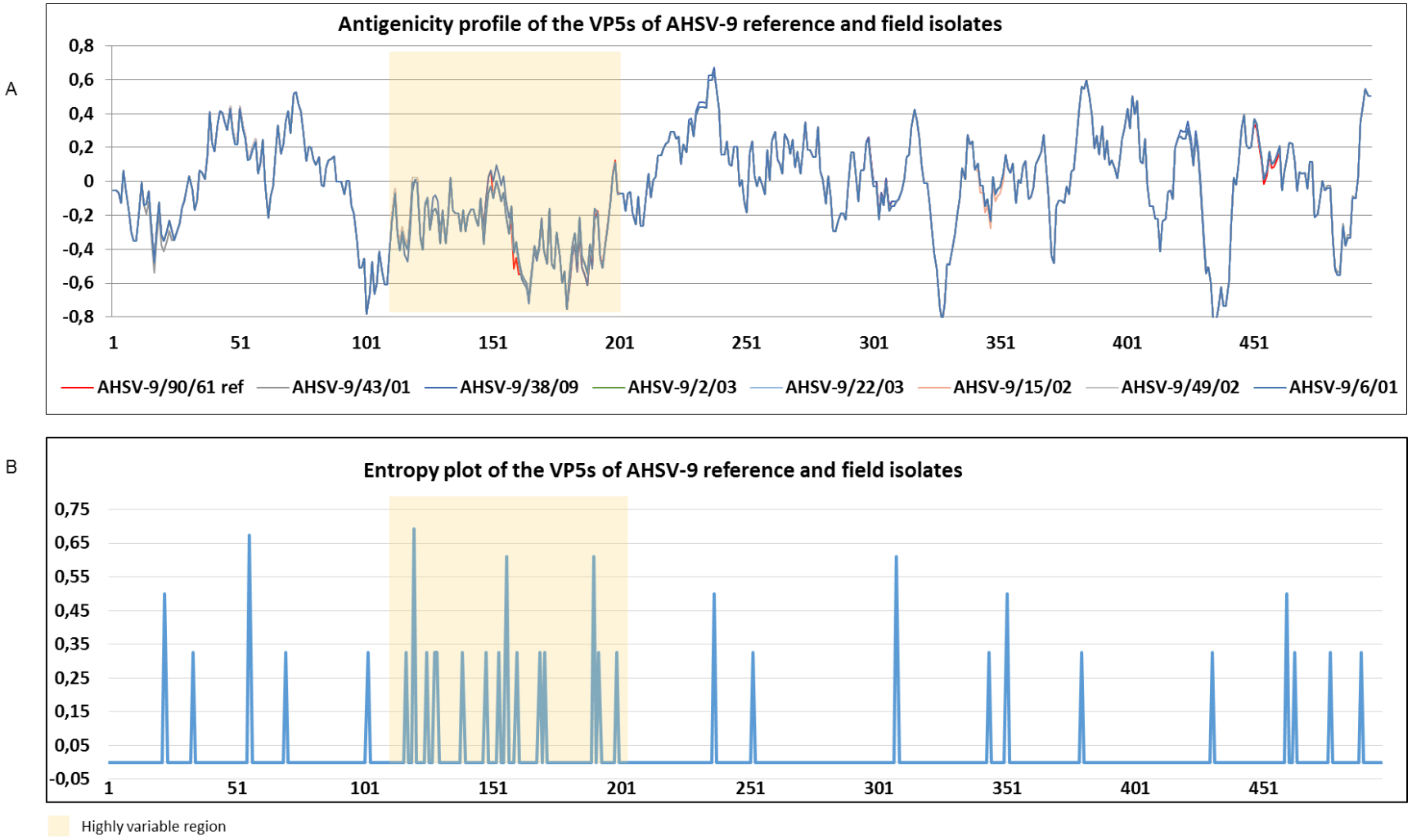

Figure 3.2: Antigenicity (A) and entropy (B) plots of the AHSV-9 VP5 proteins of the seven recent isolates and one reference strain. The region between amino acid residues 100 and 200 (highlighted in orange) contained the most variation between the virus isolates.

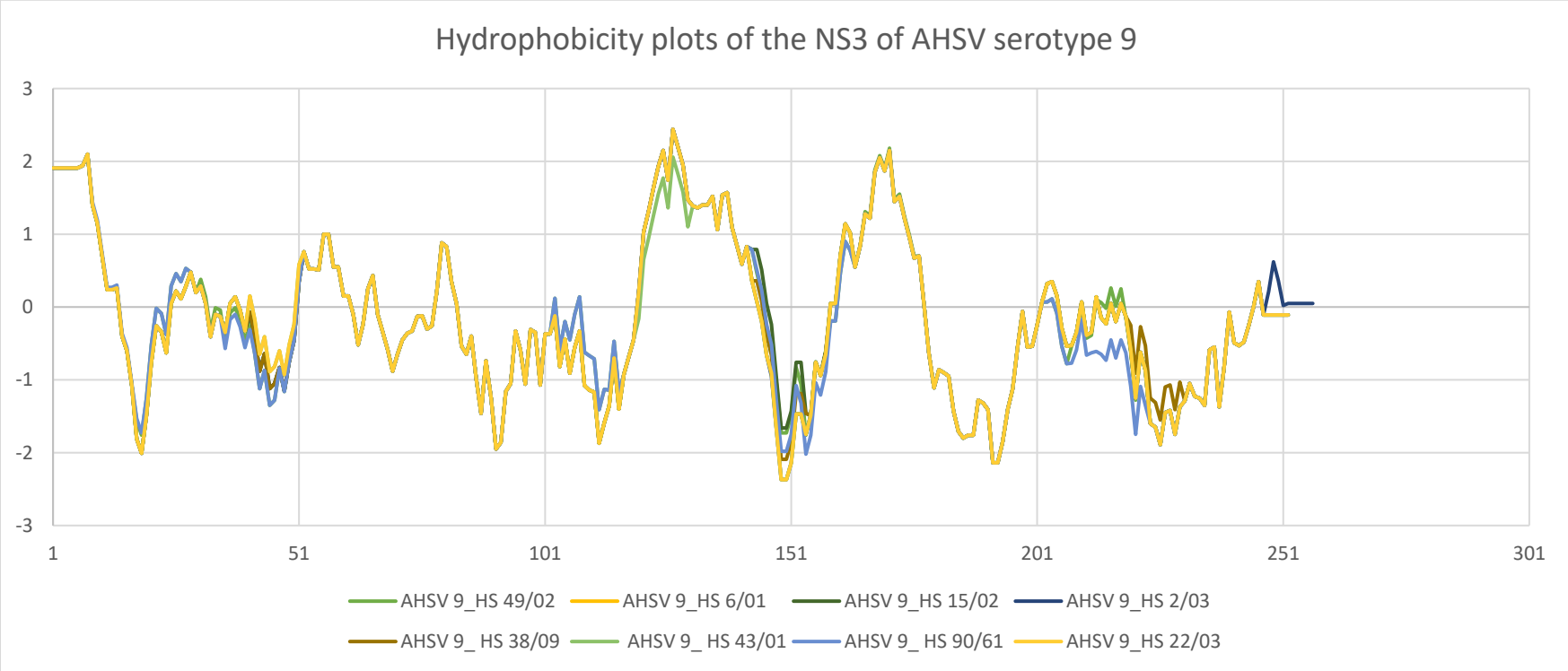

Figure 3.3: Hydrophobicity plots of the NS3 proteins of the AHSV-9 reference strain and seven AHSV-9 field isolates

3.3.4 Detection of quasispecies within the viral populations of each of the nine recent fields isolates of AHSV serotype 1-9

Detection of the low frequency mutations within the viral populations of the selected nine recent field isolates of AHSV serotypes 1-9, was completed using ultra-deep sequencing data generated in previous chapter (Section 2.2.6). Using the probabilistic variant caller, variants within the individual genome segments of the recent field isolates (2000 - 2009) were determined. Random mutations occurring in some of the genome segments of the recent isolates of AHSV were observed at various sites (Table 3.8). Except for the insertion of the G base at nucleotide base position 1636 within segment 6 of HS 68/09, all mutations observed were nucleotide base substitutions and deletions.

Table 3.8: Variants possibly depicting quasispecies within the individual genome segments of the nine recent field isolates of AHSV serotypes 1 to 9.

Sample	Serotype	Genome segment (protein)	Mutations	Count of variants	Sequencing coverage of the genome segment	Frequency (Count/coverage)
HS 107/09	1	1 (VP1)	3876 G→A	33	139	23.4
		2 (VP2)	588 G→C	18	99	18.18
		4 (VP4)	4 T→A	3	27	11.11
		3 (VP3)	704 T→C	3	21	14.29
HS 20/09	2	2 (VP2)	2071 T→C	60	153	39.22
		5 (NS1)	1415 G→A	1628	5278	30.85
		10 (NS3)	468 G→A	7744	19483	39.75
			7 A→ -	680	1068	63.67
HS 109/08	3	10 (NS3)	406 A→G	1172	5395	21.72
			396 G→T	1616	6001	26.93
			410 C→T	2981	5029	59.28
HS 68/09	4	2 (VP2)	198 G→A	160	814	19.66
			1746 G→C	160	928	17.24
			2200 T→C	427	952	44.85
		6 (VP5)	1636 - →G	29	156	18.59

Table 3.8 continued

Sample	Serotype	Genome segment (protein)	Mutations	Count of variants	Sequencing coverage of the genome segment	Frequency (Count/coverage)
HS 35/09	5	2 (VP2)	1084 T→A	85	248	34.27
		4 (VP4)	1370 T→C	429	2562	16.74
		6 (VP5)	997 G→C	1423	3490	40.77
		7 (VP6)	288 C→T	251	1635	15.35
		8 (VP7)	4 T→A	61	248	24.6
		10 (NS3)	7 A→ -	348	681	51.1
HS 63/09	6	3 (VP3)	798 T→C	356	855	41.64
		7 (VP6)	1142 C→T	45	308	14.61
			512 C→T	1487	4278	34.76
		10 (NS3)	544 A→ -	1266	8409	15.06
HS 89/09	7	1 (VP1)	1387 C→T	3	26	11.54
			1366 C→T	3	25	12
			1249 G→A	4	29	13.79
			1252 A→G	4	28	14.29
			1270 T→C	4	27	14.81
			1276 A→G	4	27	14.81
			1279 G→A	4	26	15.38
			811 T→C	12	76	15.79
			817 G→A	12	75	16
		6 (VP5)	224 G→T	3	33	9.09
			228 C→A	3	33	9.09
			170 C→T	11	83	13.25
			171 A→G	11	83	13.25
			283 C→T	4	23	17.39
			198 T→C	13	50	26
			237 T→G	14	34	41.18

Table 3.8 continued

Sample	Serotype	Genome segment (protein)	Mutations	Count of variants	Sequencing coverage of the genome segment	Frequency (Count/coverage)
HS 29/00	8	5 (NS1)	511 T→C	1238	8462	14.63
		7 (VP6)	482 T→C	1852	6726	27.53
		10 (NS3)	408 A→G	5602	9963	56.23
HS 38/09	9	3 (VP3)	1517 T→A	6	45	13.33
			1548 T→C	8	48	16.67
			1460 T→C	7	40	17.5
		7 (VP6)	622 C→T	334	2534	13.18
			341 G→A	553	4071	13.58
			551 T→A	607	3686	16.47
			641 T→A	509	3075	16.55
			58 A→G	193	1121	17.22
			580 C→T	928	3399	27.3
		9 (NS2)	484 A→T	1267	10239	12.37
			476 C→T	1262	10112	12.48
		10 (NS3)	107 T→C	4622	20568	22.47

3.4 DISCUSSION

It has been shown for a number of RNA viruses that genetic and antigenic variation occur between viral isolates (Martinez *et al.*, 1992; Takeda *et al.*, 1994; Simmonds, 2004). To date, genetic variation and similarities between the full genomes of the historic strains and the recent isolates of AHSV had not been studied. This work was aimed at determining the level of genetic variation between the available recent field isolates and the historic reference strains of AHSV, isolated four decades apart.

Inter-serotype comparative analysis of the ten genome segments' nucleotide and deduced amino acid sequences of the nine recent isolates of AHSV serotypes 1 to 9, showed that segment 2 (VP2), 6 (VP5) and 10 (NS3) were most variable across serotypes. This findings are in agreement with previously reported levels of variation for VP2, VP5 and NS3 of AHSV and BTV (Huismans *et al.*, 2004). In the analysis of other genome segments, particularly those previously specified as conserved (VP3, VP7, VP1, NS2, NS1 and VP6 genes and deduced amino acid sequences in decreasing order of conservation) (Grubman & Lewis, 1992; Maree & Huismans, 1997; Vreede & Huismans, 1998; van den Bout, 2003; Aradaib, 2009; Guthrie *et al.*, 2013), high levels of conservation were observed between serotypes. These results were expected taking into consideration the known and putative functional roles of the proteins encoded by these conserved segments during viral replication and morphogenesis, and in maintaining the overall structure of the virus. S3 and S7 as the most conserved segments, encode VP3 and VP7 respectively, and these proteins are important in the stable formation of the core and subcore layer of the virion (Roy & Noad, 2006). The high variation observed for the genome segment 2, 6 and 10 can also be explained by the functions of the encoded proteins. VP2 is responsible for the antigenic determinants of AHSV, thus contains epitopes regions of the AHSV against which a neutralising immune response is generated. VP2 is therefore subjected to selective pressure from the animal host. VP5 interacts closely with VP2 on the surface of the virion and influence the conformation of the protein, including the epitope structure (Hassan *et al.*, 2001). NS3 plays a role in viral egression from the host cell (Huismans *et al.*, 2004) and most likely has an influence on the virulence of the virus in the host animal and cell culture.

Intra-serotype comparative analysis of the corresponding segments of the nine recent AHSV field isolates serotype 1 to 9 and the nine reference AHSV strains serotypes 1 to 9, demonstrated high levels of conservation for all genome segments. With the exception of the results obtained for genome segments 2, 6 and 10 encoding for VP2, VP5, and NS3, similar levels of conservation were observed for all other segments as was observed for inter-serotype comparisons. Genome segments encoding for VP2, VP5 and NS3 were better conserved within a serotype even over four decades, with the level of conservation similar to that observed for segment 4 and 9, encoding for VP4 and VP6 respectively. Amongst the different serotypes, the most variation was observed in serotype 9, and the least in serotype 1 for comparison of corresponding segments of the recent and historic reference isolates.

Variation within isolates of AHSV, isolated four decades apart, was determined by analysis of genome segments 2, 6 and 10 encoding VP2, VP5 and NS3, respectively, of the AHSV-9 reference strain and the seven field isolates of AHSV-9 (1994 - 2009). Serotype 9 was chosen, as it showed more variation between most of the genome segments of the isolates that were compared. Aside from the fact that these were the most variable segments among the recent isolates compared in this study, in agreement with previous literature (van Niekerk *et al.*, 2001), these segments were of interest to study because of the functions of the encoded proteins. Percentage sequence identities of the nucleotide sequences were in the range of 89% - 100% for segment 2 (VP2), 93% - 100% for segment 5 (VP5) and 86% - 100% for segment 10 (NS3). The recent isolates showed to be more similar to each other than to the historical strains for these segments. This suggests that mutations in the genes accumulate over time. The observed genetic variation between the recent and historic strain could, however, mostly be due to the geographical separation of the isolates. The AHSV-9 historical strain used in this study was obtained from the republic of Chad whereas the recent isolates were all from South Africa. This is due to the fact that prior to and around the 1960s, AHSV-9 had not been reported to circulate in Southern Africa. Previous studies on AHSV have shown a correlation between the level of the variation and place of origin or the geographical location of an outbreak. A study of AHSV NS3 sequences of isolates from southern

African, showed that isolates of different geographical location are more variable as compared to those originating from the same region (van Niekerk *et al.*, 2001).

Non-synonymous amino acid substitutions were observed within the VP2, VP5 and NS3 sequences of seven recent field isolates, relative to the reference strain, from the multiple sequence alignments of the amino acid sequences of the proteins. For VP2, these substitutions corresponded with changes in the antigenicity profiles and the predicted secondary structures of the proteins. It is worth noting that some of the substitutions were identified within the neutralising epitope regions of VP2 and the surrounding regions (Martínez-Torrecuadrada & Casal, 1995; Bentley *et al*., 2000; Martínez-Torrecuadrada *et al*., 2001). Similar studies in other arboviruses have shown the significance of amino acids substitutions on the viral proteins associated with eliciting a protective immunity and resulting in antibody neutralisation of the virus (Labuda *et al.*, 1994; Brault *et al.*, 2004). Jiang *et al.* (1993) reported on the neutralisation escape mutants of the Louping ill (LI) virus, which differed from the non-mutant viruses by single amino acid substitutions within the E glycoprotein. The E glycoprotein of the flaviviridae is a major structural protein which shows hemagglutinin (HA) activity and induces HA inhibiting, neutralizing and protective antibodies.

A variable region between amino acid residues 100 - 201 was identified within the VP5 sequences of the seven recent isolates and the reference strain. The region corresponded with high entropy values from the entropy plot of the VP5 proteins confirming the region to be variable. These findings are in agreement with those previously reported for VP5 of BTV. In the study of BTV VP5 proteins, three variable regions that were identified were amino acid residues 120 - 190, 273 - 345 and 410 - 481 (Gould & Pritchard, 1988; Oldfield *et al*., 1991; Singh *et al.*, 2004). It is unclear as to why amino acid residues 100 - 201 shows the most variation for the AHSV and BTV. It can be speculated that this may be the region of protein that is not under any selective pressure to remain unchanged. The rest of the AHSV VP5 protein which is much more hydrophilic may be under some pressure/constrain to remain conserved. This view agrees with those shared by other workers that the conserved regions of the VP5 protein may be structurally constraint not to change due to important interaction with VP2 and VP7 (Gould &

Pritchard, 1988). The identified variable region (amino acid residue 100 - 201) was highly hydrophobic. These results are in agreement with those reported by Hassan *et al*. (2001), who also reported that two amphipathic helices on the C-terminus are important for the function of the VP5 protein.

In the analysis of NS3 sequences of the seven recent isolates of AHSV-9 and the reference strain of AHSV-9, a number of non-synonymous amino acid substitutions within the hydrophobic domain 1 (HD1) and the region between HD1 and hydrophobic domain 2 (HD2) were identified. These findings agreed with those reported by other workers on NS3 for AHSV and BTV, which indicated that HD1 and the region between the hydrophobic domains are the most variable between isolates (van Niekerk *et al.*, 2001). Minor differences in the hydrophobicity profiles of the NS3 proteins were observed, and these differences correlated with the observed amino acid substitutions within the HD1 regions of the recent field isolates, with the NS3 sequences of the recent isolates being either more or less hydrophobic at various points. In a study performed by Huismans *et al.* (2004), NS3 was shown to be cytotoxic and that the HD1 and HD2 regions of the protein are needed for the NS3 cytotoxic property in AHSV infected-mammalian cells. In rotaviruses, it has been demonstrated that a single amino acid substitution within the NSP4 protein (enterotoxin associated with virulence in rotavirus) enhanced the cytotoxicity of the NSP4 protein in infected cells (Tian *et al.*, 2000). Hence, the amino acid substitutions that were observed in this study in the NS3 protein sequences of the recent field isolates may affect the cytotoxicity of the NS3 proteins of the recent isolates in infected cells, and therefore the virulence of the viruses.

Recent isolates (2000 - 2009) of AHSV prepared from equine spleen or blood with a recorded passage history in Vero and BHK21 cells were subjected to ultra-deep sequencing, and their genomes analysed for quasispecies at the genome segments level. The sequencing reads obtained were mapped to the consensus sequences of the individual genome segments to identify the variants possibly depicting quasispecies. The observed mutations included substitutions, additions and deletions of single nucleotide bases. These results confirm the error-prone replication process of dsRNA viruses which contribute greatly to the emergence of quasispecies. The obtained results were expected,

as it has been reported that RNA viruses exist as heterogeneous populations of closely related genomes referred to as quasispecies, with the variants showing great fitness under existing conditions being selected for (Domingo & Holland, 1997). It is postulated that, genetic drift occurring during sequential passage of BTV in animal host and insect vector, also contribute to the quasispecies observed in nature (Bonneau *et al.*, 2001). In addition, new viral variants can be fixed in nature by random feedings of affected animals by the arthropod vectors thereby further contributing to the variation observed in individual segments of the virus.

AHSV and other viruses in the *Orbivirus* genus are arboviruses. Hence, their transmission between susceptible animal hosts is mediated by arthropod vectors (du Toit, 1944). The level of genetic variation therefore observed in AHSV and other arboviruses is expected to be lower by comparison to viruses that infect a single host, such as influenza viruses and the human immunodeficiency virus (HIV). These viruses have been reported to evolve rapidly (Gorman *et al.*, 1992; Wain-Hobson 1996). AHSV is therefore required to infect and replicate in the insect and animal hosts, allowing as few changes possible. This will ensure that the virus retains its fitness and does not become extinct (Steele & Nuttal, 1989; Scott *et al.*, 1994). It is has been argued that this explains the slow evolving rate of most arboviruses in nature.

It has been indicated that BTV and AHSV evolve under strong purifying selection (Carpi *et al.*, 2010; MacLachlan & Guthrie, 2010). As a result of the negative purifying selection pressures acting on AHSV and that, the AHSV has an RNA genome lacking error corrective mechanism during replication, it is tempting to conclude that the variations observed in this study between the historic reference strains and recent field isolates are not as high as expected. However, we identified a number of non-synonymous amino acid substitutions within and around the epitopes regions of AHSV VP2 protein. Predicted secondary structures of the proteins indicated that these changes could potentially alter the structure of VP2 within the infected cells. Future studies therefore should focus on the virus neutralisation assays of the recent field isolates against antiserum produced with reference strains to determine the effect of the mutation observed between the isolates.

Chapter 4: Concluding remarks

This study aimed to characterise the genomes of the recent field isolates and complete inter- and intra-isolate comparative analysis of the recent isolates against the historic strains. To achieve this aim dsRNA from recent field isolates was amplified by sequence-independent cDNA synthesis and ultra-deep sequencing was completed. The consensus nucleotide sequences of the individual genome segments were determined by *de novo* assembly and by mapping of sequence reads to individual genome segments sequences obtained from GenBank. Comparative comparisons were completed at nucleotide and amino acid level.

Successful extraction of pure dsRNA was achieved and each of the ten segments could be converted to cDNA and sequenced using established methods (Chapter 2). The results from the ultra-deep sequencing provided high read counts and the data that was of a high enough quality to be able to determine accurately the consensus sequences for each isolate and to detect evidence of quasispecies.

The consensus nucleotide sequences of the individual genome segments for the recent field isolates were compared to that of the historical strains. The level of variation for the three most variable proteins of AHSV VP2, VP5 and NS3, was also determined using multiple isolates of a single serotype (AHSV-9). Pairwise and multiple sequence alignments of the individual genes, their deduced amino acid sequences and percentage sequence identities indicated that there is variation between the isolates (recent and historical) with the observed maximum variability of 28% and 9.5% on nucleotide and amino acid level, respectively. The results obtained were expected and consistent with the nature of the RNA containing AHSV and its mode of transmission. RNA viruses show more variation than DNA viruses due to the fact that they lack the proof-reading mechanism during replication (Domingo & Holland, 1997). However, AHSV like other arbovirus is transmitted between hosts by an arthropod vector, therefore allowing minimum changes such that its fitness in the two environments is not compromised (Steele & Nuttal, 1989; Scott *et al.,* 1994).

Mutations observed within the VP2, VP5 and NS3 amino acid sequences of the seven recent isolates and one reference isolate of the same serotype (AHSV-9), correlated with changes in the predicted secondary structure of VP2, the hydrophobicity and or antigenicity profiles of the proteins and also showed high entropy for VP2 and VP5. Comparison of the segments encoding for VP3, VP7, NS1, NS2 and NS3 of the recent and reference isolates, showed percentage sequence identity at > 86% suggesting that PCR-based diagnostic assays making use of these segments remain relevant over four decades.

In conclusion, although a relatively small number of recent AHSV field isolates were included in this study, the complete genome sequences obtained were informative, enabling the identification of noticeable sequence changes. By comparison to the reference AHSV strains that were isolated over four decades ago, we were able to identify significant non-conservative amino acid mutations within the three most variable AHSV proteins, which correlated with changes in their predicted secondary structures. However, in future studies, comparative genome analysis of more recent isolates of each AHSV serotype may yield more insight into the extent of the genetic divergence that occurred between the AHSV reference and field strains that are currently circulating. Thus, determination of the complete genome sequences of more field isolates that can be included in the database and analyses will be beneficial. The aim of this study was not to investigate genetic divergence between the reference strains and field isolates collected subsequently at regular time intervals. Thus, only AHSV field isolates collected between 2000 and 2009 were included in this study. Nevertheless, in our view, the results of such a study are unlikely to differ significantly from those obtained in this study and we were interested mainly in determining the level of genetic divergence between the AHSV reference and recent isolates.

This study was, therefore, the first step in the investigation of the evolutionary dynamics of AHSV. It is certain that there is minimal variation between the isolates of AHSV found in the field. It is, however, unknown if other evolutionary processes such as recombination and reassortment occur. In future, studies should, therefore, focus on generating sequence data from more field isolates of all nine serotypes of AHSV to determine

whether recombination and reassortment occur in AHSV and its contribution to the variation observed between the isolates of AHSV. Serological and structural studies should also be completed to determine the significance of the changes observed within VP2, VP5 and NS3. This will enable us to determine whether the currently used vaccine and diagnostic tests, which are based on the historical strains of AHSV, require an update.

Congress contributions

Mokotoane, R., Koekemoer, O., Ngoveni, H., van Schalkwyk, A. & Maree, S., Complete genome comparison of nine recent and historic field isolates of African horse sickness virus, 12th International dsRNA Virus Symposium 2015, 6 - 10 October, Goa, India.

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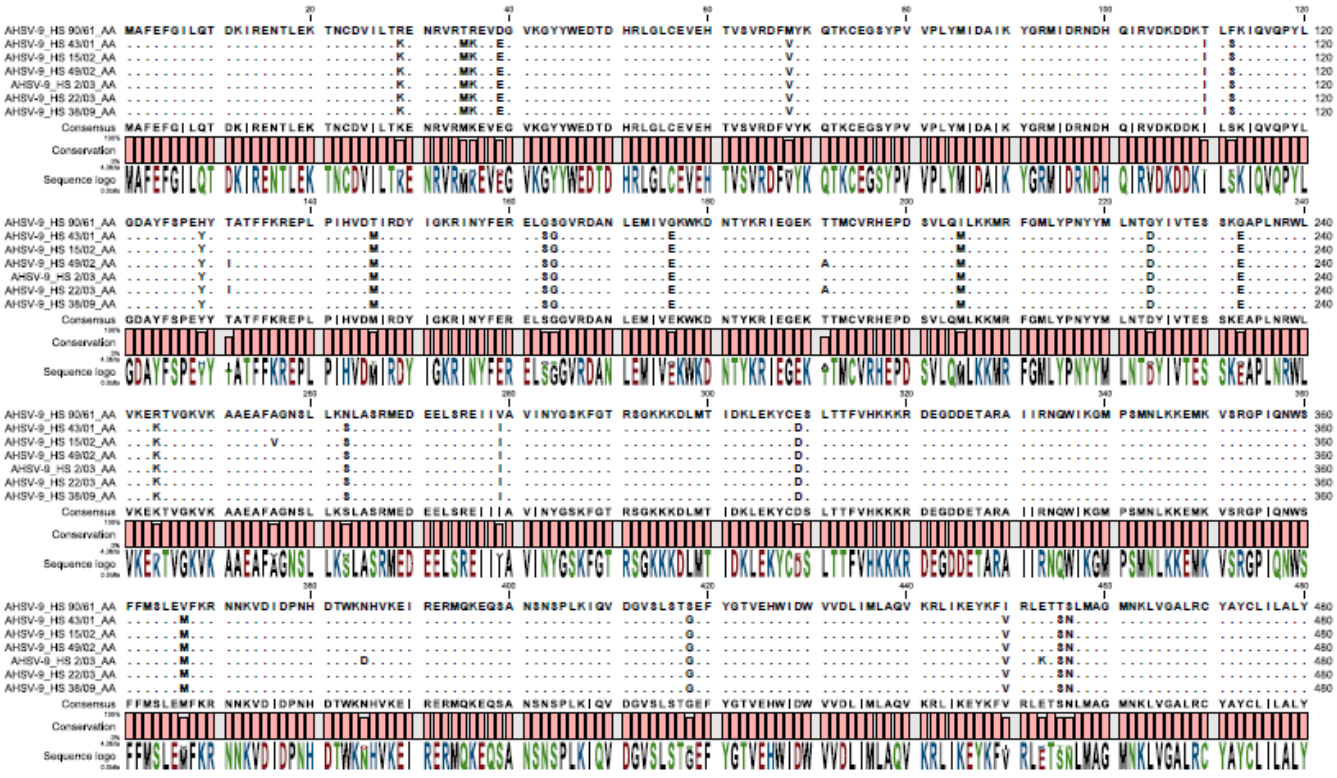
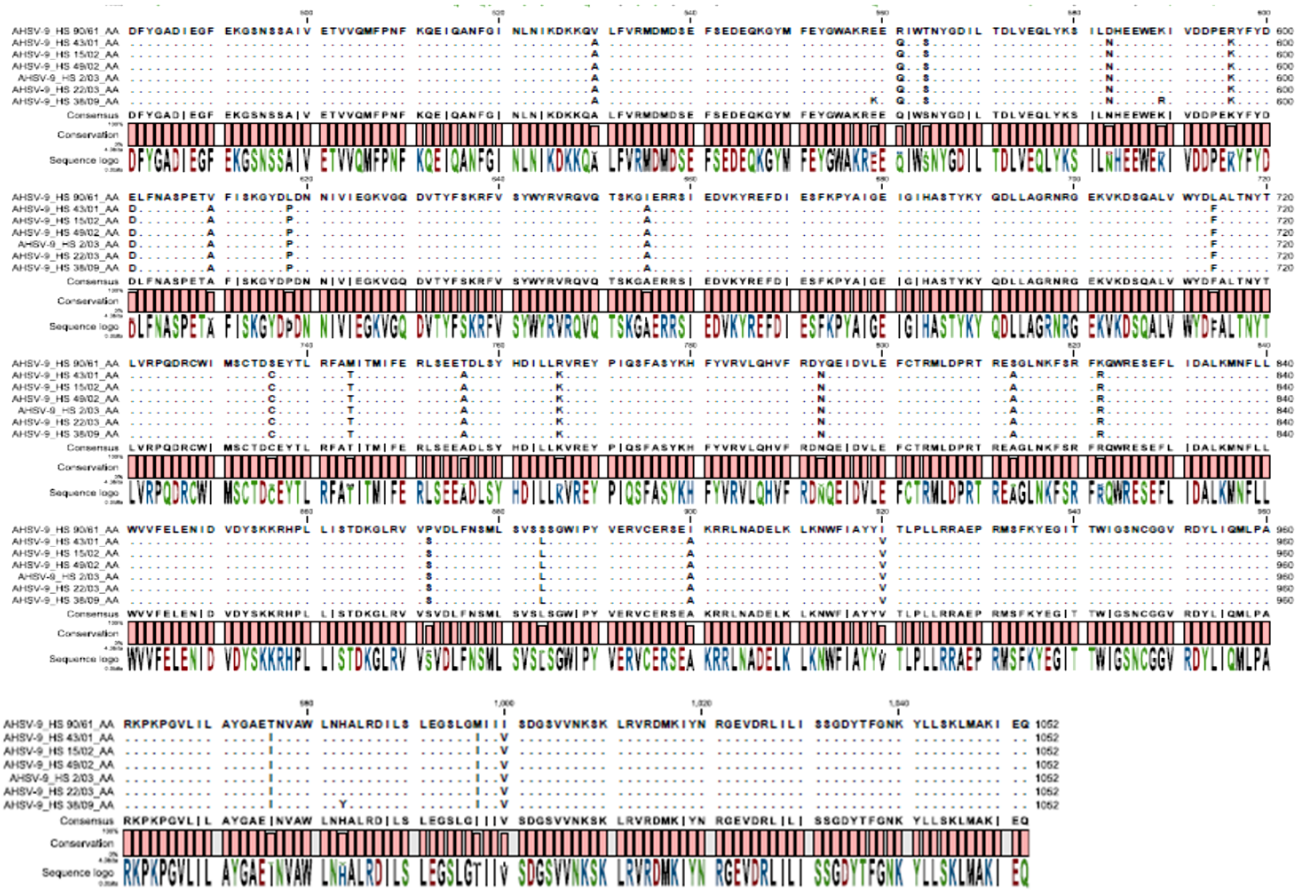
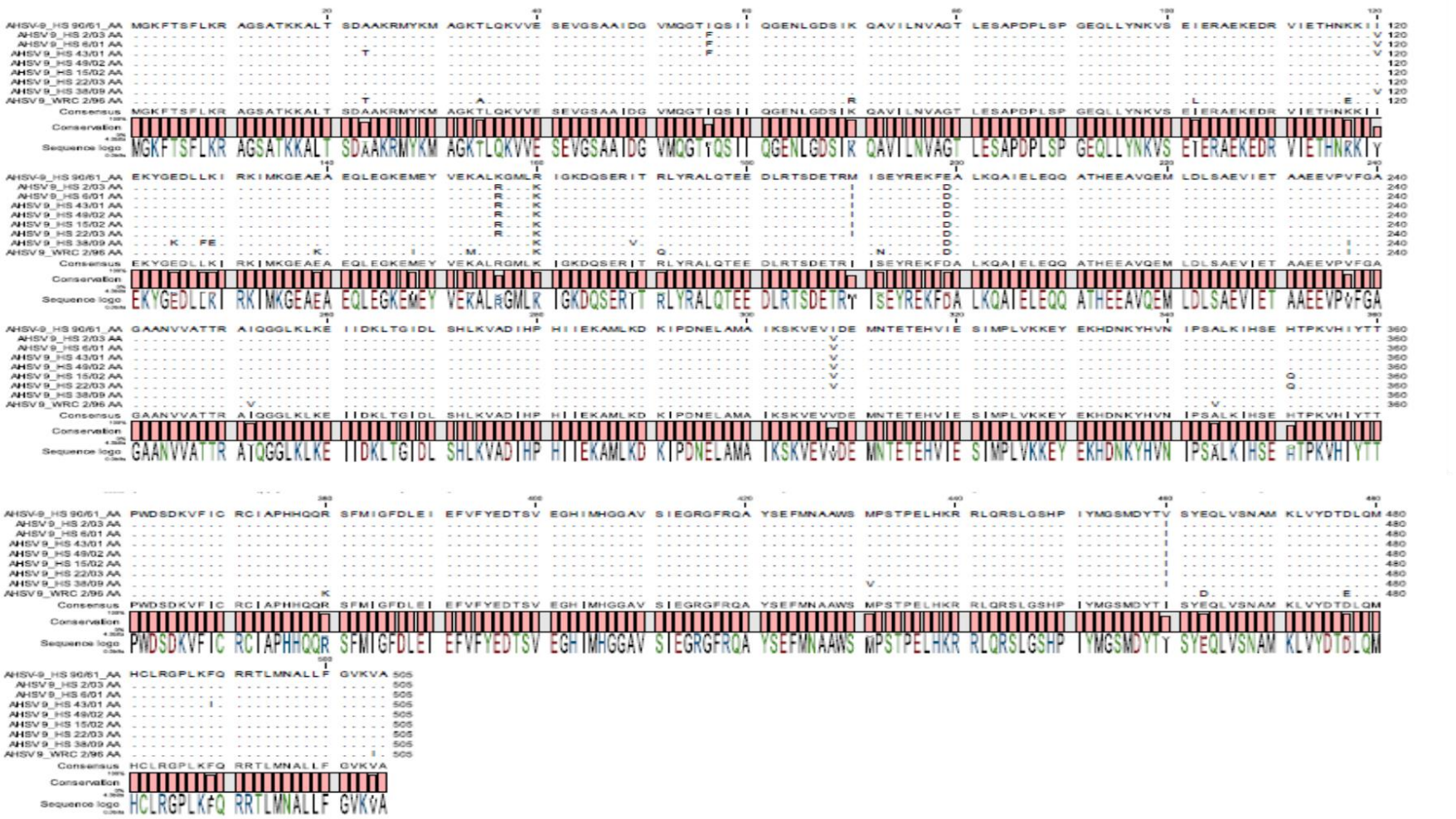
Appendix A: Multiple amino acids sequence alignments of VP2, VP5 and NS3



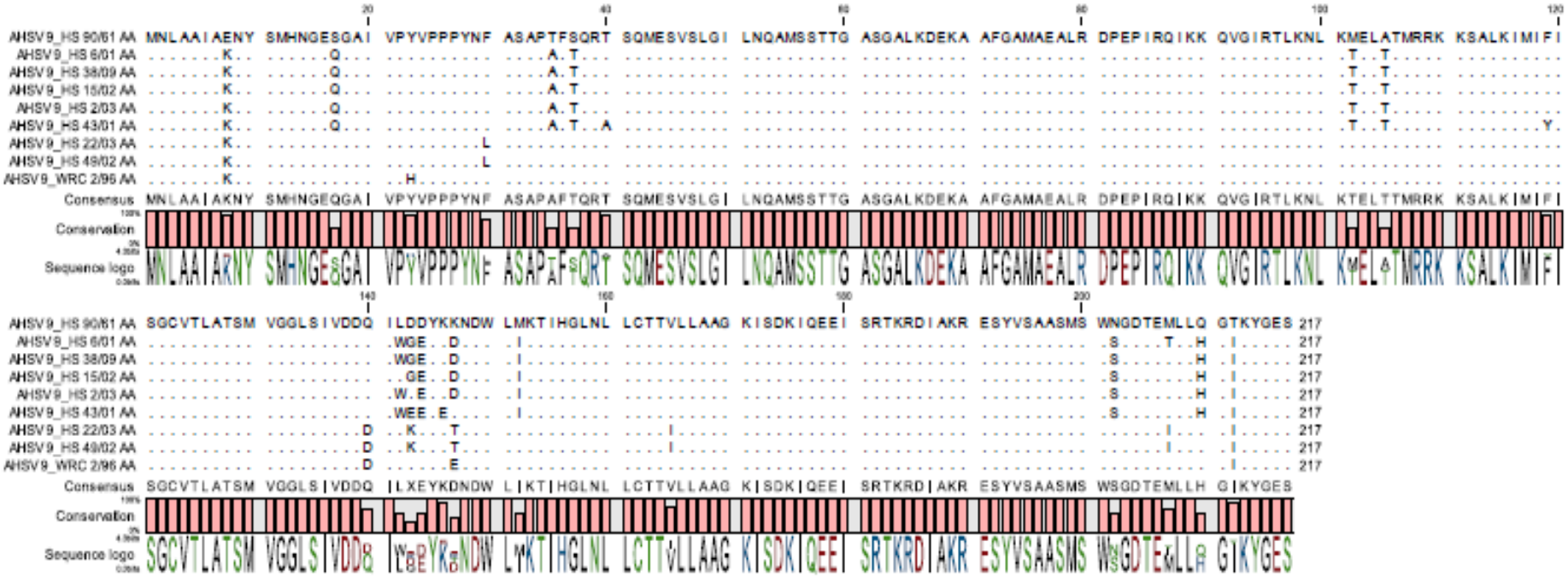
A1 - The alignment of the deduced amino acid sequences of segment 2 of AHSV. The dots in the alignment denote amino acid residues identical to the AHSV 9_90_61 reference strain. The bases highlighted in red indicate change of amino acid in the field and recent strains that is different in property. A consensus amino acid sequence and conservation graph are observed below the alignment.



A1 - *Continued*



A2 - The alignment of the deduced amino acid sequences of segment 5 of AHSV. The dots in the alignment denote amino acid residues identical to the AHSV 9_90_61 reference strain. A consensus amino acid sequence and conservation graph are observed below the alignment.



A3 - The alignment of the deduced amino acid sequences of segment 10 of AHSV. The dots in the alignment denotes amino acid residues identical to the AHSV 9_90_61 reference strain. A consensus amino acid sequence and conservation graph are observed below the alignment.

Appendix B: Amino acid substitutions within VP2, VP5 and NS3 of recent field isolates

B1 - Amino acid substitutions within the VP2 amino acid sequences of the field isolates as compared to the reference strain (AHSV9_ HS 90/61) amino acid sequence. Non-conservative amino acid substitutions are indicated in bold.

Position (Amino acid in reference)	AHSV 9_HS 43/01	AHSV 9_HS 15/02	AHSV 9_HS 49/02	AHSV 9_HS 2/03	AHSV 9_HS 22/03	AHSV 9_HS 38/09
29 Arg	Lys	Lys	Lys	Lys	Lys	Lys
35 Thr	Met	Met	Met	Met	Met	Met
36 Arg	Lys	Lys	Lys	Lys	Lys	Lys
39 Asp	Glu	Glu	Glu	Glu	Glu	Glu
68 Met	Val	Val	Val	Val	Val	Val
110 Thr	Ile	Ile	Ile	Ile	Ile	Ile
112 Phe	Ser	Ser	Ser	Ser	Ser	Ser
129 His	**Tyr**	**Tyr**	**Tyr**	**Tyr**	**Tyr**	**Tyr**
131 Thr			Ile			
146 Thr	Met	Met	Met	Met	Met	Met
163 Gly	Ser	Ser	Ser	Ser	Ser	Ser
164 Ser	Gly	Gly	Gly	Gly	Gly	Gly
176 Gly	**Glu**	**Glu**	**Glu**	**Glu**	**Glu**	**Glu**
191 Thr			Ala			
205 Ile	Met	Met	Met	Met	Met	Met
224 Gly	**Asp**	**Asp**	**Asp**	**Asp**	**Asp**	**Asp**
233 Gly	**Glu**	**Glu**	**Glu**	**Glu**	**Glu**	**Glu**
244 Arg	Lys	Lys	Lys	Lys	Lys	Lys
256 Ala		Val				
263 Asn	Ser	Ser	Ser	Ser	Ser	Ser
279 Val	ile	ile	ile	ile	ile	ile
309 Glu	Asp	Asp	Asp	Asp	Asp	Asp
367 Val	Met	Met	Met	Met	Met	Met

B1 continued - Non-conservative amino acid substitutions are indicated in bold.

Position (Amino acid in reference)	AHSV 9_HS 43/01	AHSV 9_HS 15/02	AHSV 9_HS 49/02	AHSV 9_HS 2/03	AHSV 9_HS 22/03	AHSV 9_HS 38/09
385 Asn	**Asp**	**Asp**	**Asp**	**Asp**	**Asp**	**Asp**
418 Ser	Gly	Gly	Gly	Gly	Gly	Gly
450 Ile	Val	Val	Val	Val	Val	Val
453 Glu				**Lys**		
455 Thr	Ser	Ser	Ser	Ser	Ser	Ser
456 Ser	Asn	Asn	Asn	Asn	Asn	Asn
530 Val	Ala	Ala	Ala	Ala	Ala	Ala
559 Glu						Lys
561 Arg	**Gln**	**Gln**	**Gln**	**Gln**	**Gln**	**Gln**
564 Thr	Ser	Ser	Ser	Ser	Ser	Ser
583 Asp	**Asn**	**Asn**	**Asn**	**Asn**	**Asn**	**Asn**
589 Lys						Arg
596 Arg	lys	lys	lys	lys	lys	lys
601 Glu	Asp	Asp	Asp	Asp	Asp	Asp
610 Val	Ala	Ala	Ala	Ala	Ala	Ala
618 leu	Pro	Pro	Pro	Pro	Pro	Pro
655 Ile	Ala	Ala	Ala	Ala	Ala	Ala
714 Leu	Phe	Phe	Phe	Phe	Phe	Phe
736 Ser	Cys	Cys	Cys	Cys	Cys	Cys
744 Met	Thr	Thr	Thr	Thr	Thr	Thr
756 Thr	Ala	Ala	Ala	Ala	Ala	Ala
766 Arg	Lys	Lys	Lys	Lys	Lys	Lys
793 Tyr	Asn	Asn	Asn	Asn	Asn	Asn
813 Ser	Ala	Ala	Ala	Ala	Ala	Ala
822 Lys	Arg	Arg	Arg	Arg	Arg	Arg
872 Phe	Ser	Ser	Ser	Ser	Ser	Ser
884 Ser	Leu	Leu	Leu	Leu	Leu	Leu
900 Ile	Ala	Ala	Ala	Ala	Ala	Ala
920 Ile	Val	Val	Val	Val	Val	Val
976 Thr	Ile	Ile	Ile	Ile	Ile	Ile
983 His					**Tyr**	**Tyr**
997 Met	Ile	Ile	Ile	Ile	Ile	Ile
1000 Ile	Val	Val	Val	Val	Val	Val

B2 - Amino acid substitutions within the VP5 amino acid sequences of the field isolates as compared to the reference strain (AHSV9_ HS 90/61) amino acid sequence. Non-conservative amino acid substitutions are indicated in bold.

Position (Amino acid in reference)	AHSV 9_HS 2/03	AHSV 9_HS 6/01	AHSV 9_HS 22/03	AHSV 9_HS 43/01	AHSV 9_HS 49/02	AHSV 9_HS 15/02	AHSV 9_HS 38/09
23 Ala				Thr			
56 Ile	Phe	Phe	Phe	Phe			
120 Ile	Val	Val	Val	Val			Val
125 Glu							Lys
128 Leu							Phe
129 Lys							Glu
156 Lys	Arg	Arg	Arg	Arg	Arg	Arg	
169 Ile							Val
190 Met	Ile	Ile	Ile	Ile	Ile	Ile	
199 Glu	Asp	Asp	Asp	Asp	Asp	Asp	Asp
237 Val							Ile
308 Ile	Val	Val	Val	Val	Val	Val	
351 His						**Gln**	
431 Met							Val
460 Val	Ile	Ile	Ile	Ile	Ile	Ile	Ile
489 Phe				Ile			

B3 - Amino acid substitutions within the NS3 amino acid sequences of the field isolates as compared to the reference strain (AHSV9_ HS 90/61) amino acid sequence. Non-conservative amino acid substitutions are indicated in bold.

Position and AA in reference	AHSV 9_HS 6/01	AHSV 9_HS 38/09	AHSV 9_HS 15/02	AHSV 9_HS 2/03	AHSV 9_HS 22/03	AHSV 9_HS 43/01	AHSV 9_HS 49/02
8 Glu	Lys	Lys	Lys	Lys	Lys	Lys	Lys
17 Ser	Gln	Gln	Gln	Gln	Gln	Gln	
30 Phe							Leu
35 Thr	Ala	Ala	Ala	Ala	Ala	Ala	
37 Ser	Thr	Thr	Thr	Thr	Thr	Thr	
40 Thr					Ala	Ala	
102 Met	Thr	Thr	Thr	Thr	Thr	Thr	
105 Ala	Thr	Thr	Thr	Thr	Thr	Thr	
119 Phe						Tyr	
140 Gln							Asp
142 Leu	Trp	Trp		Trp	Trp	Trp	
143 Asp	**Gly**	**Gly**	**Gly**		Glu	Glu	Lys
144 Asp	Glu	Glu	Glu	Glu	Glu	Glu	
146 Lys					Glu	Glu	
147 Lys	Asp	Asp	Asp	Asp			**Thr**
152 Met	Ile	Ile	Ile	Ile	Ile	Ile	
165 Val							Ile
202 Asn	Ser	Ser	Ser	Ser	Ser	Ser	
207 Met	Thr						Ile
210 Gln	His	His	His	His	His	His	
212 Thr	Ile	Ile	Ile	Ile	Ile	Ile	Ile