DEVELOPMENT OF A PROTOCOL FOR THE MOLECULAR SEROTYPING OF THE AFRICAN HORSE SICKNESS VIRUS

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(BScHons)

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ABSTRACT

African horse sickness (AHS) is a viral disease with high mortality rates, vectored by the *Culicoides* midge and affecting members of the Equidae family. AHS is endemic to South Africa, and, as a result, affects export and international competitiveness in equine trade, and impacts significantly on the South African racehorse and performance horse industries. AHS also has devastating consequences for rural and subsistence equine ownership. The protocol developed in this dissertation has the potential to serotype and confirm the AHS virus within a few hours at significantly less cost than current methods. It will ease the financial and time constraints of studying an outbreak in real time and has the potential to solve many of the unknown factors surrounding AHS, particularly and most importantly, the role that each serotype plays in outbreaks and the form of the disease contracted by horses.

This dissertation focuses on preliminary investigations into the development of an assay, and provides a brief analysis of a preliminary set of melt curves from the nine serotypes. The AHS virus was propagated in both embryonated chicken eggs and Vero cell culture. The latter resulted in the most reliable viral RNA extraction. Of the two genome segments that provide divergence among the nine serotypes of AHS, Segment 2 was found to be less suitable in a bioinformatic analysis for primer design. Segment 10 is more conserved and was selected for primer design for reverse transcription-PCR of AHS viral RNA. Primers were designed using GenBank, ClustalX2, Primaclade, TreeView and POLAND and successfully amplify the 10-190 bp region of Segment 10 of all nine AHS viral serotypes. High Resolution Melt (HRM) analysis was performed on the amplified products using the Corbett Rotor-Gene® 6000. HRM is based on the release of an intercalating fluorescent dye that is released from DNA as it is denatured with increasing temperature immediately following PCR. The melt curves were grouped into three distinct ‘bins’, which correspond to the groupings of a dendrogram based on the sequenced products. This constitutes proof of concept for a protocol for the molecular serotyping of the AHS virus. This provides a strong platform for the validation of a rapid diagnostic assay for informing vaccine programs, while advancing epidemiological modelling of African horse sickness.

Preface

The experimental work described in this dissertation, unless otherwise stated, was carried out in the School of Agricultural Sciences and Agribusiness and the Molecular Biology Unit in the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Pietermaritzburg, from January 2008 to December 2009, under the supervision of Ms. Marion B. Young and co-supervision of Dr. Gregory M. F. Watson.

Candidate's Declaration

I, Shaun Reinder Groenink declare that

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I hereby release this dissertation for examination in my capacity as supervisor.

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December 2009

I hereby release this dissertation for examination in my capacity as co-supervisor.

Dr. Gregory M. F. Watson
Molecular Biology Unit
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December 2009

TABLE OF CONTENTS

List of Figures

List of Tables

Acknowledgements

“Be still, and know that I am God’

Psalm 46:10

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Lastly, this dissertation is dedicated to the countless many horses, donkeys and mules that have and will succumb to the dreadful, horrifying disease that is African horse sickness. One day we may go some way to eliminating the virus from large parts of Africa.

SOMEWHERE *(Author unknown)*

Somewhere, somewhere, in God's own space, there must be some sweet, pastured place

Where creeks sing on, and tall trees grow; some paradise where horses go.

For by the love that guides my pen, I know great horses live again.

List of Abbreviations

AHS	African horse sickness
AHSV	African horse sickness virus
bp	base pair(s)
BSL	Biosafety level
DNA	deoxyribonucleic acid
DoA	Department of Agriculture, Forestry and Fisheries
ds	double-stranded
ECE	embryonated chicken eggs
EMEM	Eagle's Minimum Essential Medium
FBS	foetal bovine serum
HRM	High Resolution Melt
IPA	isopropyl alcohol
MWM	molecular weight marker
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
OBP	Onderstepoort Biological Products
OIE	World Organisation for Animal Health
OVI	Onderstepoort Veterinary Institute
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SNT	Serum Neutralisation Tests
ss	single-stranded
$TCID_{50}$	50% tissue culture infective dose
TBE	Tris-Borate-EDTA buffer
VN	Virus Neutralisation

Chapter 1: INTRODUCTION AND PROPOSAL

African horse sickness (AHS) is a viscerotropic, non-contagious, vector-borne disease with high mortality and morbidity that primarily affects members of the Equidae taxon (OIE, 2007). There are nearly 300,000 horses and approximately 160,000 donkeys that make up the South African national herd (Gerdes, 2006). According to data from South African National Parks and KZN Wildlife, there were over 30,000 zebra in protected areas around the country. Worldwide, it is estimated that there may be up to 100 million working equines, the majority in developing countries (El Idrissi & Lubroth, 2006). Due to climate change, the vector appears to be spreading, increasing the number of equines at risk (Mellor & Hamblin, 2004). Thousands of horses are lost every year in South Africa due to AHS. AHS influences export and international competitiveness in equine trade, and impacts significantly on the South African racehorse and performance horse industry (Agüero *et al.*, 2008). The current vaccine produced by Onderstepoort Biological Products (OBP) remains the best and most practical means of protection. However, the vaccine cannot be relied upon for full protection from infection. The individual response may vary; there may be some interference between the serotypes in the polyvalent vaccine or over-attenuation of some of the vaccine strains, leading to weakly immunogenic vaccine strains (Coetzer & Erasmus, 1994). As an alternative, inactive, monovalent vaccines have been proposed, which many researchers acknowledge are far safer and more effective (Mellor & Hamblin, 2004). New generation monovalent vaccines have also been developed (Guthrie *et al.*, 2009).

An accurate and rapid diagnosis of equine infectious diseases is an important goal for researchers and veterinarians alike. Early detection of causative agents and identification of strain/serotype has immediate benefits that include applying the correct treatment regime for the animal, notifying authorities in the case of notifiable diseases and implementing suitable control measures to prevent further spread of the disease. The development of rapid assay methods to identify AHS serotypes is imperative in the study of the disease, in order that control (including vaccination) might augment the conventional prophylactic strategies currently employed. The ability to serotype an AHS outbreak rapidly, combined with the increasing development of monovalent vaccines, makes a rapid serotyping assay even more important (Koekemoer *et al.*, 2000). As a result, the national equine population could be protected far more effectively against AHS. Furthermore, the serotyping and classification of the virus will assist greatly in a

rapid classification of future outbreaks for taxonomic and epidemiological purposes. A rapid assay may also promote future exportation of South African equine athletes and improve the livelihood of rural people who depend on equines as working animals for subsistence.

African horse sickness (AHS) accounts for large numbers of equine deaths per year, although many go undetected and unreported (AHS-Trust, 2008). There are a great number of rural horses and other equids used for subsistence agriculture and other tasks in previously disadvantaged communities that contract the disease and die without ever being reported to the authorities, due to a lack of education or intervention by the authorities (AHS-Trust, 2009, *Personal Communication*). Furthermore, the sero-prevalence of the virus and the vaccination coverage in the South African equid population are unknown (Lord *et al.*, 1997a) and remain so to this day. These problems are compounded by the lack of research funding for AHS. Expensive tests are required to understand the disease thoroughly and are therefore not performed, with many owners unwilling to pay for tests on a dead horse. Tests to determine the serotype are undertaken only at Onderstepoort Veterinary Institute, take up to two weeks to achieve a result and cost in excess of R1000. All of these factors compound the minimal number of epidemiological studies being undertaken which lead to often grossly misunderstood aspects of the disease. A rapid assay will go some way to easing the financial and time constraints of studying an outbreak in real time. This has the potential to solve many of the unknowns surrounding AHS, particularly and most importantly, the role that each serotype plays in outbreaks and the form of the disease contracted by horses. In addition, the strain is not usually classified until sometime after the initial outbreak, as no system to rapidly classify the virus involved in the outbreak exists. Early identification of the serotype will benefit overall investigations and our understanding of the clinical disease (Abdalla *et al.*, 2002). The last two decades have seen a shift in the pursuit for rapid diagnostics from classical microbiology to molecular biology based techniques, such as nucleic acid detection, particularly the polymerase chain reaction (PCR). The applicability of PCR has increased dramatically with the advent of real-time or quantitative PCR (Pusterla *et al.*, 2006). The system that is proposed is based on DNA amplification and High Resolution Melt (HRM) analysis that will detect the AHS virus and serotype it in a single test in a few hours and could potentially cost under R50 per sample.

The significance of this research lies within the advantages of PCR combined with High Resolution Melt analysis (HRMA) for detecting the AHS virus in the blood or other biological samples from infected equids. Noteworthy advantages exist in using PCR and HRMA in clinical situations where conventional microbiology is inadequate, time-consuming or labour-intensive (e.g. cell culture), or difficult and hazardous (Abdalla *et al.*, 2002). The rapid nature of PCR also has important consequences for limiting the spread of highly contagious pathogens in an epidemic. Results are available in less than 24 hours as opposed to 4-5 days for virus isolation (Stone-Marschat *et al.*, 1994).

High resolution melting (HRM) was introduced in 2002 through the collaborative efforts of academia (University of Utah) and industry (Idaho Technology) (Reed *et al.*, 2007). A variety of methods had previously been developed to detect the DNA sequence variation of PCR products, however, these involve extra processing and separation steps subsequent to the PCR run, include additional apparatuses and are time-consuming. Gundry *et al*. (2003) described the ability of melt temperatures to distinguish unique variants in a homogenous, closed-tube procedure performed automatically after PCR. Reed *et al.* (2007) considers HRM the simplest method of determining sequence variation.

HRM requires normal PCR reagents, a fluorescing double-stranded DNA (dsDNA)-binding dye and a short period of closed-tube, post-PCR analysis (Reed & Wittwer, 2004). In its simplest form, HRM involves the release of an intercalating fluorescent dye from dsDNA as it is denatured or dissociated into single-stranded DNA (ssDNA) with increasing temperature. The melt curve is generated from heating the sample through a range of temperatures, as fluorescence data is continuously collected. As the temperature is increased, the fluorescence drops rapidly at a characteristic point, indicating the dissociation of the dsDNA into single strands and the release of the bound dye. This point, the melting temperature of the DNA (T_m), is dependent on GC content, length and sequence. This behaviour results in a characteristic melt profile for each individual amplicon (Reed *et al.*, 2007). The decrease in fluorescence is analysed *in silico* (Corbett, 2006). HRM has a number of applications including mutation detection, genotyping and species identification (Gundry *et al.*, 2003; Corbett, 2006). HRM is simple, cost-effective and requires no post-PCR processing, such as agarose gel electrophoresis. It also compares favourably with other similar, expensive techniques (Corbett, 2006). The sensitivity of HRM is evident in its ability to detect the

smallest genetic change such as single base changes (single nucleotide polymorphisms) (White & Potts, 2006). In general, the greater the number of changes, the easier they are to detect using HRM. Developing technically simple and reliable methods for detecting sequence variations in related genes has become an important goal of molecular diagnostics. Although DNA sequencing still remains the "gold standard", it is too labour- and time-intensive for clinical, routine use (Highsmith, 2004).

A review of the literature pertaining to HRM reveals that most applications have sought the detection of single point mutations. However, in 2006, HRM analysis successfully and repeatedly differentiated between species of the amoebaflagellate genus *Naegleria* using a single primer set (Robinson *et al.*, 2006). The amplified sequences were almost invariant in individual species, but divergent among species. The melt curves that resulted were distinguishable and unique for each species, due to the differences seen in the positions and relative heights of the peaks on the melt curves. Feline caliciviruses, *Plasmodium* species and *Cryptosporidium* species have also all been successfully species-differentiated using HRM analysis (Helps *et al.*, 2002; Tanriverdi *et al.*, 2002; Mangold *et al.*, 2005). HRM analysis was also used to detect and identify clinically important bacteria and used to distinguish *Bacillus anthracis* species (Cheng *et al.*, 2006; Fortini *et al.*, 2007). Most recently, HRM was used to successfully distinguish *Chlamydophila psittaci* genotypes and fowl adenovirus serotypes (Mitchell *et al.*, 2009; Steer *et al.*, 2009).

The African horse sickness virus has nine serotypes, each with ten common double-stranded RNA segments making up the genome (Grubman & Lewis, 1992). Two of the segments are responsible for the genetic diversity amongst the serotypes (Roy *et al.*, 1994; Venter *et al.*, 2000) and it these segments that will be investigated and used to distinguish the different serotypes using HRM. Segment 2 (encoding for the VP2 protein) and Segment 10 (encoding for the NS3 protein) have been selected as possible targets for the development of this assay. VP5, an additional outer capsid protein may also be involved in serotype determination (Koekemoer *et al.*, 2000). Segment 10 is being investigated in this dissertation as it is more conserved than Segment 2. In addition, there is more sequence "depth" on current, available databases for Segment 10, which inevitably impacts the choice of segment.

In the future, rapid serotyping assays, such as the one proposed, will gain increasing significance as new-age vaccines are developed. These vaccines are likely to be monovalent (Mellor & Hamblin, 2004) and as such, the serotype of the infecting virus must be determined before the correct monovalent vaccine can be used. “Since protection against African horsesickness (AHS) is serotype-specific, rapid serotyping of AHSV is crucial to identify the correct vaccine serotype for efficient control of the spread of AHS outbreaks, especially when they occur in non-endemic regions” (Koekemoer & van Dijk, 2004). The South African national equine herd will enjoy enhanced protection from each annual outbreak as the serotype and classification of the virus will be determined rapidly and the correct vaccine program can immediately be undertaken.

Internationally, in non-endemic zones, it will become crucial to serotype the virus as rapidly as possible so that the correct monovalent vaccine can be distributed to protect equines in the surrounding areas. In the past, delays in determining the serotype saw the rapid spread of the disease to unprotected equines and a decrease in the ability to control the outbreak. “The amount of damage that a foreign animal disease will cause is directly proportional to the time between introduction and accurate diagnosis” (Brown, 2002). In the case of African horse sickness, which has nine distinct serotypes, control of the disease depends on a roll-out of the correct monovalent vaccine. Rapid serotyping is therefore imperative. Additionally, the use of HRMA combined with PCR adds a new dimension to classification and the use of taxonomic keys. In a sense, the melt curve generated provides the taxonomic key to enable researchers to correctly classify the virus. This combination of techniques has the potential to be expanded to other organisms and possibly provides an additional dimension to taxonomic keys in biological systems.

Use of PCR and HRM analysis will make epidemiological studies into African horse sickness more thorough. Indeed, the modern nucleic-acid based assays have revolutionised the diagnosis of disease and its related epidemiological studies. These molecular systems have made the isolation of pathogens secondary. The characterisation of pathogens will follow this lead and other isolation steps may become obsolete (Eaton & White, 2004). This study represents the future in that regard. The cost and analysis time for serotyping will be drastically reduced. Blood from sub-clinical equids can provide differential diagnoses for early-warning system

strategies and interventions to be employed. These include early quarantining of viral 'hot spots'; subunit, recombinant, vectored vaccination (Guthrie *et al.*, 2009; Young, 2009, *Personal Communication*) and prophylaxis measures can be extrapolated to vector control and husbandry measures (Jenkins, 2008; Simpkin, 2008). Furthermore, the possibility of real-time simulation modelling on AHS becomes a very real possibility as the role that different serotypes play in the epidemiology of the disease will lead to a comprehensive modelling strategy that will predict future outbreaks and so permit a more effective, monovalent vaccination program.

It is expected that with the advent of new-age vaccines that are easier to administer, with far fewer side effects and complications, and a rapid serotyping program at the beginning of localised outbreaks, all segments of the local horse population will be far better protected. The impact of this research could also be extended to taxonomic and classification systems of all organisms as a 'rapid confirmation' of field samples. It has already been noted that comprehensive molecular databases should be established in order to monitor the spread of the virus during an outbreak and increase movement controls (Gerdes, 2006). A database of melt curves would augment this.

However, some caveats do exist. Knowledge of the molecular systems of pathogenesis and virulence and the nucleic acid based approach to serotype determination is rudimentary and this must be borne in mind when attempting to rapidly detect, identify and characterise viral RNA. Live viral cell culture will still be necessary. Despite the rapidity and multiplexing abilities of PCR and related technologies, they are unable to detect actual, live viral particles (Eaton & White, 2004).

The overall approach to the development of this protocol will be to test a variety of primer pairs on a culture of each serotype of the virus, to determine which portion of the sequence will give the most divergent melt curves, whilst using the least number of primer pairs to achieve that purpose. Presently, only one primer pair has been tested to provide proof of concept. Segment 2 will be analysed at a later stage and the results from all primer pairs will be compared to determine which will give the most reliable identification of a particular serotype.

This work has already been acknowledged as intellectual property and a provisional patent (Application No. 2009/04542; June, 2009) has been filed through the Intellectual

Property and Technology Transfer division of the Research Office of the University of KwaZulu-Natal.

Chapter 2: LITERATURE REVIEW

2.1 Introduction

African horse sickness (AHS) is a viscerotropic, non-contagious, viral disease with high mortality and morbidity that primarily affects members of the Equidae family (OIE, 2007). The African horse sickness virus (AHSV) (NCBI Taxonomy ID: 40050) is a double stranded RNA orbivirus of the Reoviridae family (Mellor & Hamblin, 2004; OIE, 2004a). Other names for the disease include Perdesiekte, Pestis Equorum, La Pesta Equina and Peste Equina Africana. AHSV is also considered an arbovirus as it 'can infect haematophagous arthropods after the blood from an infected vertebrate has been ingested. It multiplies in the arthropod's tissues and is transmitted by bite to other susceptible vertebrates' (Mellor, 2000). Mortality in horses may reach 95% and in donkeys 50%. Due to its severity and ability to spread rapidly, AHS is considered as one of the most fatal diseases that affect equids and is listed by the World Organisation for Animal Health (OIE) (previously List A) (Mellor & Hamblin, 2004; OIE, 2009).

2.2 History

The first recorded epidemic of African horse sickness was in 1327 in the Yemen, although the disease almost certainly originated from Africa (Coetzer & Erasmus, 1994; Mellor & Hamblin, 2004). In Africa, a monk named Father Monclaro first described the disease from a 1569 account of journeys into central and east Africa, using Indian horses (Theiler, 1921). In South Africa, the disease first appeared in horses when they were brought to the Cape of Good Hope in 1652 by the Dutch East India Company (Coetzer & Erasmus, 1994). Only 60 years after the introduction of horses to the Cape did the first major, officially recorded, outbreak occurred in 1719 where 1700 horses died. It was, however, already apparent that frost appeared to retard the spread of the disease (Theiler, 1921; Mellor & Hamblin, 2004). The most severe recorded outbreak of AHS was in 1854/55 when it was estimated that over 70,000 horses, or 40% of the horse population, died from AHS. By 1921, reports of AHS outbreaks in Northern Rhodesia (Zambia), South West Africa (Namibia), Angola, British East Africa (Kenya), German East Africa (Tanzania), Zanzibar, Uganda, the Sudan, Abyssinia (Ethiopia) and Eritrea were described (Theiler, 1921).

In the early 1900's, the cause of the disease was found to be a virus by the work of pioneers into AHS research (Coetzer & Erasmus, 1994). At the same time it was becoming apparent to Theiler (1921) that immunologically distinct strains existed. This theory gained further credibility with the work of Alexander in the 1930's. Alexander also started work that would have important vaccine-related consequences when he showed that the virus was attenuated during passage in chicken egg embryos (Coetzer & Erasmus, 1994). In 1944, it was confirmed that the AHS virus was vectored by *Culicoides imicola* (du Toit, 1944). Nine serotypes have been described, the last being in 1960 (Howell, 1962). Historically, it is apparent that major outbreaks of the disease occur approximately every 20-30 years. However, a decline in the zebra and horse population and the advent of the polyvalent vaccine produced by Onderstepoort Biological Products, in the last century, has largely prevented the massive outbreaks of the past, although outbreaks continue to have devastating consequences in certain, more localised areas (Coetzer & Erasmus, 1994; Mellor & Hamblin, 2004).

2.3 Aetiology

2.3.1 Taxonomy

African horse sickness is caused by the African horse sickness virus (AHSV). The agent was first described as an ultravisible organism (Theiler, 1921). AHSV is a member of the Reoviridae family and is classified in the *Orbivirus* genus. Other closely related *Orbiviruses* include the bluetongue (BTV) and equine encephalosis viruses (EEV) (Spence *et al.*, 1984; Mellor & Hamblin, 2004; OIE, 2004a). Orbiviruses were initially described in 1971 (in Gorman, 1979) to define arthropod-borne viruses with distinctive morphologies and physically and chemically identical characteristics. Reoviridae have a double-stranded RNA (dsRNA) genome encapsulated by a single viral particle that is quasi-spherical and displays icosahedral symmetry (Gorman, 1979). AHSV is an arbovirus. It is transmitted biologically to vertebrates through blood-sucking arthropod vectors, namely the biting midges of the *Culicoides* genus (Mellor, 2000; Kuno & Chang, 2005). Theiler (1921) alluded to the presence of more than one type of the virus as equines supposedly immune to the virus could become fatally re-infected. Nine immunologically distinct serotypes have since been found to exist, numbered 1-9, the last being isolated by Howell in 1960 (Howell, 1962; Hamblin *et al.*, 1991). The lack of any new serotypes being discovered since is indicative of a

genetically stable virus with little to no antigenic changes (Howell, 1962; Erasmus, 2004). No complete cross neutralisation is evident between any two strains although there is a degree of cross neutralisation between serotypes 6 and 9 (Howell, 1962) and between serotypes 5 and 8 (Mellor & Hamblin, 2004). Field evidence suggests that there is no intratypic variation (Coetzer & Erasmus, 1994).

2.3.2 AHSV Properties

The 68-70 nm diameter virion (Figure 2.1) is unenveloped with a double-layered capsid consisting of 32 capsomeres (Gorman, 1979; Bremer *et al.*, 1990; Erasmus, 2004) with the outer capsid more diffuse and less discernable (Wood, 1973). The outer layer of the *Orbivirus* genus of viruses appears to be conserved (Nason *et al.*, 2004) and the physico-chemical properties of the virion appear to be common to all Orbiviruses (Gorman, 1979; Mellor & Hamblin, 2004). The virus is relatively heat-stable, but can be inactivated at 50°C for 3 hours and 60°C for 15 minutes. It can survive at 37°C for 37 days (OIE, 2002). Moreover, the virus may maintain its infectivity if isolated from putrid blood (Theiler, 1921). Infected blood samples may still yield virions for up to a year if stored at 4°C (House *et al.*, 1990). The virus can survive in the pH range of 6.5 – 8.5, and is more sensitive to acidity than to alkalinity (Erasmus, 2004; Mellor & Hamblin, 2004). AHSV is inactivated by ether, ß-propiolactone or formalin (OIE, 2002).

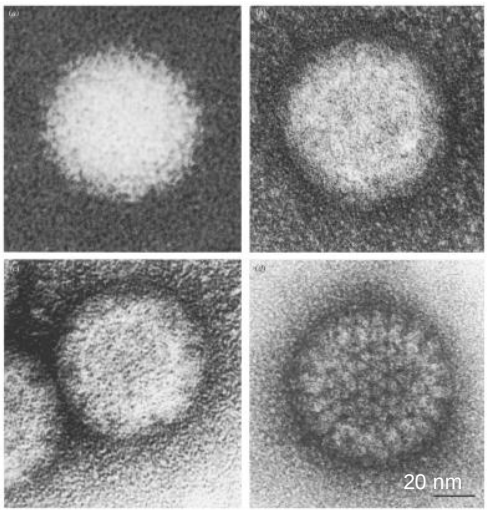

Figure 2.1 A selection of electron micrographs of AHS viral particles purified by centrifugation through different gradients. Top left: Sucrose. Top right: CsCl. Bottom left: Infectious subviral particles, sucrose. Bottom right: Cores (after Burroughs *et al.*, 1994).

2.3.3 Structure

AHSV is a double-stranded RNA virus made up of 10 genome segments (Grubman & Lewis, 1992). This was first demonstrated when the RNA was analysed on polyacrylamide gels resulting in 10 bands (Gorman, 1979; Spence *et al.*, 1984). Orbiviruses contain segmented genomes, which may allow for gene re-assortment and antigenic diversity. However this is contradictory to earlier reports that AHSV is a very stable virus (Howell, 1962). Segment numbers were assigned on the basis of molecular weight data (Spence *et al.*, 1984; Bremer *et al.*, 1990) and are numbered in order of their migration (Roy *et al.*, 1994). However, there is some discrepancy in the literature and amongst published sequences with regard to coding assignments of segments 6, 7, 8, 9 and 10. This is due to the fact that the segments are of almost identical molecular weight, hindering their differentiation on gel systems (Table 2) (Bremer *et al.*, 1990; Quan, *Personal Communication*). The coding assignments of Grubman and Lewis (1992) have segment 6 coding for VP5 and VP6 and an additional protein, NS4 for Segment 10. However, this view is an isolated one and is incompatible with systems used for the bluetongue virus, the prototype orbivirus (Burroughs *et al.*, 1994). The two proteins encoded in segment 6 have since been found to be related, the smaller one being a truncated version of the larger one (Roy *et al.*, 1994). Table 2.1 gives the coding assignments from four sources for comparative purposes. For the purposes of this dissertation, the coding assignments of Mertens *et al.* (2006) shall be used.

Table 2.1: Coding assignments of Grubman & Lewis (1992), Roy *et al.* (1994), Quan (2008) and Mertens *et al.* (2006) for the ten genome segments of the African horse sickness virus (AHSV).

Genome Segment	Protein Nomenclature			Size (bp) (Mertens *et al.*, 2006)
	(Grubman & Lewis, 1992)	(Roy *et al.*, 1994; Quan, *Personal Communication*)	(Mertens *et al.*, 2006)	
1	VP1	VP1	VP1	3965
2	VP2	VP2	VP2	3205
3	VP3	VP3	VP3	2792
4	VP4	VP4	VP4	1978
5	NS1	NS1	NS1	1748
6	**VP5 / VP6**	**VP5**	**VP5**	1566
7	**VP7**	**VP7**	**VP6**	1169
8	**NS2**	**NS2**	**VP7**	1167
9	**NS3**	**VP6**	**NS2**	1166
10	**NS4/NS4a**	**NS3/NS3a**	**NS3/NS3a**	756

The core particle is composed of the major proteins (VP3 and VP7) and the minor proteins (VP1, VP4 and VP6) and encloses the 10 genome segments. (Bremer *et al.*, 1990; Roy *et al.*, 1994; Maree *et al.*, 1998). Together, these proteins make up the serogroup-specific epitopes (Mellor & Hamblin, 2004). The outer capsid is made up of VP2 and VP5 (Roy *et al.*, 1994) (Figure 2.2).

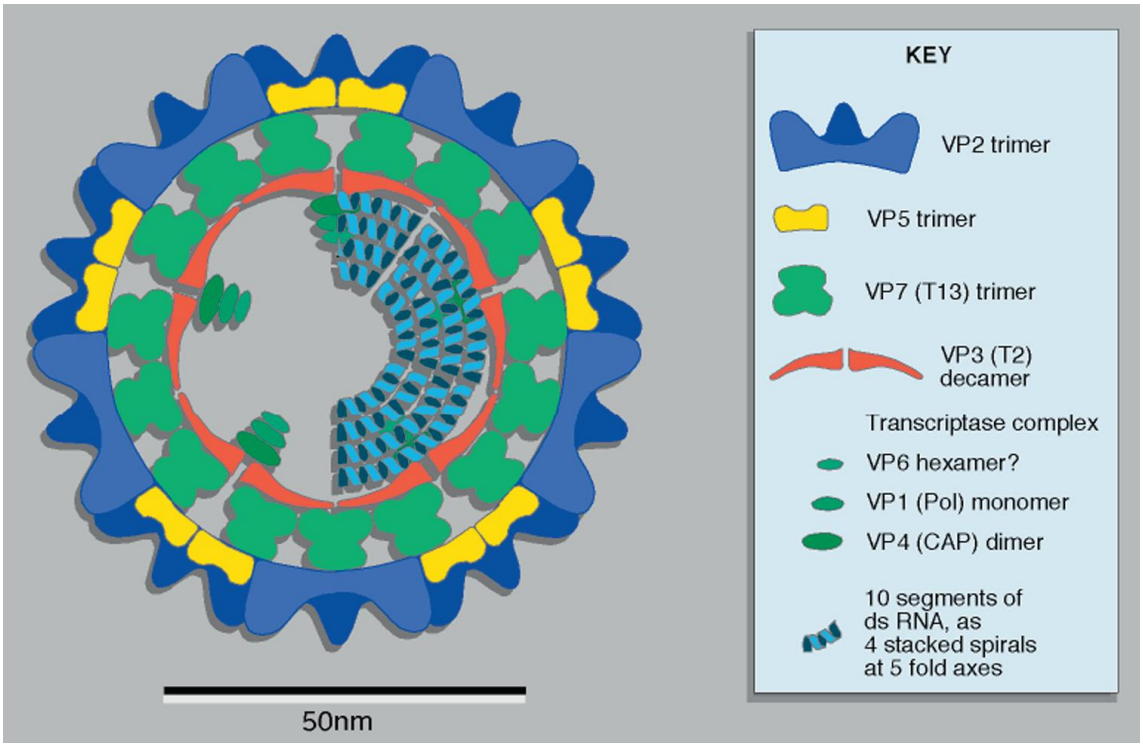

Figure 2.2: Schematic diagram of the structure of an orbivirus showing the organisation of the major and minor structural proteins (after Wilson *et al.*, 2009).

VP2 is the most variable among the nine serotypes (Burrage & Laegreid, 1994) with almost 90% of the sequences varying between 46% and 52% on a pair-wise alignment in one study (Huismans *et al.*, 2004). As such, it is responsible for serotype diversity and specificity (Roy *et al.*, 1994; OIE, 2004a). VP2 and VP5 are together responsible for virus neutralisation activity (OIE, 2004a). In addition to the above structural or viral proteins (VP), three non-structural proteins are present, namely NS1, NS2 and NS3/NS3a (Laviada *et al.*, 1995; Mellor & Hamblin, 2004) and are mainly concerned with enzymatic activities (Table 2.2). It has recently been found that after VP2, NS3 is the second most variable protein (Venter *et al.*, 2000).

Table 2.2: Data derived from a number of sources describing the accepted functions of the ten proteins common to all orbiviruses and their molecular weight where available.

Protein	*Molecular Weight (kDa)*	*Function/s*
VP1	150	RNA-directed RNA Polymerase
VP2	122-124	Outer capsid structure Serotype-specificity Receptor binding Haemagglutinating activity Host-specific immunity Cell attachment, virus penetration
VP3	103	Inner capsid structure
VP4	34	Guanylyl transferase RNA capping
VP5	57	Outer capsid - structure Destabilisation of endocytosed vesicle membranes Cell penetration
VP6	38-46	Helicase
VP7	38	Major component of inner core
NS1	63	Major component of tubules in host cell
NS2	41	Form inclusion bodies in host cells; ssRNA affinity
NS3/3a	24	Possibly involved in the release of virions from cell

(after Gorman, 1979; Huismans *et al.*, 1987; de Sá *et al.*, 1994; Roy *et al.*, 1994; Martinez-Torrecuadrada *et al.*, 1996; Turnbull *et al.*, 1996; Maree & Huismans, 1997; Maree *et al.*, 1998; Vreede *et al.*, 1998; Potgieter *et al.*, 2003; Huismans *et al.*, 2004; Maree & Paweska, 2005; Bhattacharya *et al.*, 2007; von Teichman & Smit, 2008; Chiam *et al.*, 2009; Potgieter *et al.*, 2009).

2.3.4 AHSV Replication

Once the virus has entered a mammalian cell, it removes VP2 and VP5. The removal of these two outer proteins causes the RNA polymerase to activate with an associated loss in infectivity (Gorman, 1979). The core structure that remains is transcriptionally active and protects the genome from host cell detection. Replication occurs in the cytoplasm of the host cell after the virion has depressed host cell protein synthesis (Gorman, 1979). However, bluetongue virus particles have been found under the cell surface as well as on the surface.

Tubules and inclusion bodies are formed by NS1 and NS2 respectively. It is believed that NS2 may be involved in requisitioning core proteins and single stranded viral RNA into the inclusion bodies. The minor proteins (VP1, VP4 and VP6) form the transcriptase complex that is encapsulated by VP3. This subcore acts as a foundation for VP7 proteins to attach and form the stable core structure. It is not known at what point VP5 and VP2 attach to the subcore to complete the virus particle. NS3 appears to interact with VP2 to facilitate virus release. (Wood, 1973; Spence *et al.*, 1984; Bhattacharya *et al.*, 2007). In studies done on the bluetongue virus, it was found that the virus binds to glycophorins in human and porcine erythrocytes (Eaton & Crameri, 1989).

2.3.5 Dendrogram Analysis

Relationships between serotypes of the AHS virus have not been studied to a large degree. The first dendrogram analysis was on Segment 10 (de Sá *et al.*, 1994). Serotypes 1, 4 and 8 were sequenced and compared to previously sequenced serotypes 3 and 9. Serotypes 1 and 8 appeared to be closely related, as well as serotypes 4 and 9. Serotype 3 was more closely related to serotypes 4 and 9 than serotypes 1 and 8. (Figure 2.3)

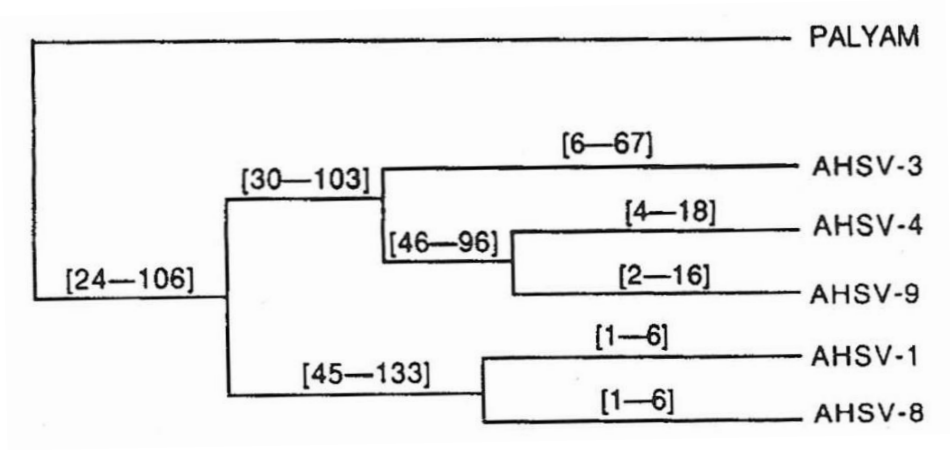

Figure 2.3: Relationships between serotypes 1, 3, 4, 8 and 9 of AHSV Segment 10. Preliminary dendrogram relationships based on the coding sequences of Segment 10. Tree length = 379, consistency index = 0.868, values above branches correspond to branch lengths under all character optimisation. Palyam represents another virus of the orbivirus group (de Sá *et al.*, 1994).

With the above sequence alignment, it was found that Segment 10 of the AHS virus is far more variable than Segment 10 of the bluetongue virus or equine encephalosis virus.

2.3.6 AHS Virus Serotypes

The role of serotypes in the epidemiology of AHS is poorly understood. The relationship between serotypes, geographical distribution, virulence and transmission is all unknown. One of the first studies looked at the basic reproduction number, R_0, in an attempt to understand the implication of serotypes on the epidemiology of the disease. R_0 is a measure of the probability of transmission of a pathogen. R_0 can be used in the calculation of control strategies such as vaccination and the coverage required to halt an epidemic. However, R_0 depends on the independence of the transmission of each serotype. It also depends on whether an infection of one serotype affects the infection of a second serotype.

A study conducted on zebras found that the distribution of serotypes was non-independent, implying some sort of relationship between the serotypes that has yet to be elucidated. Cross-immunity, biting rates, spatial and temporal variations and genetic susceptibilities may all be responsible. A very high R_0 is indicative of the likely differences in infectivity of different serotypes for midges or zebras or both (Lord *et al.*, 1996a; Lord *et al.*, 1997b). As far as immunity to the different serotypes is concerned, a

horse that recovers from an infection from a particular serotype develops a life-long immunity to that serotype, but may remain susceptible to others (Mellor, 1993). A strain of AHSV that occurred in Kenya in the early 1990s was not neutralised by antiserum of any of the nine previous serotypes leading to speculation that a tenth serotype may exist (Mellor, 1993). However, only nine recognised serotypes exist today, which may indicate the genetic stability of the virus. Table 2.3 shows the nine recognised serotypes accepted as reference strains.

Genetic re-assortment of the genome is always a concern when dealing with viruses. Segmented genomes, as exist in orbiviruses, are thought to facilitate genetic re-assortment. New types may therefore arise if a cell is co-infected. However, Howell in 1966 observed that only one strain of the bluetongue virus was ever found in infected sheep over a number of years (Gorman, 1979). This, combined with the fact that no additional AHSV serotypes have been described since the 1960's, lends itself to the idea that the AHS virus is genetically stable.

Table 2.3: AHSV Serotypes and historical isolates/reference strains (Ozawa & Dardiri, 1970)

Serotype	Historical name
1	A501
2	OD
3	L
4	Vryheid
5	VH
6	114
7	Karen
8	18/60
9	S2

The distribution of serotypes in South Africa is not well studied. A map of the serotype distribution from the 2008/2009 outbreak is given in Figure 2.4. Not all samples received by the Department of Agriculture are sent for serotyping due to expense and time. As a result, it is unlikely that the distribution map can be viewed as a correct representation of the actual situation and distribution.

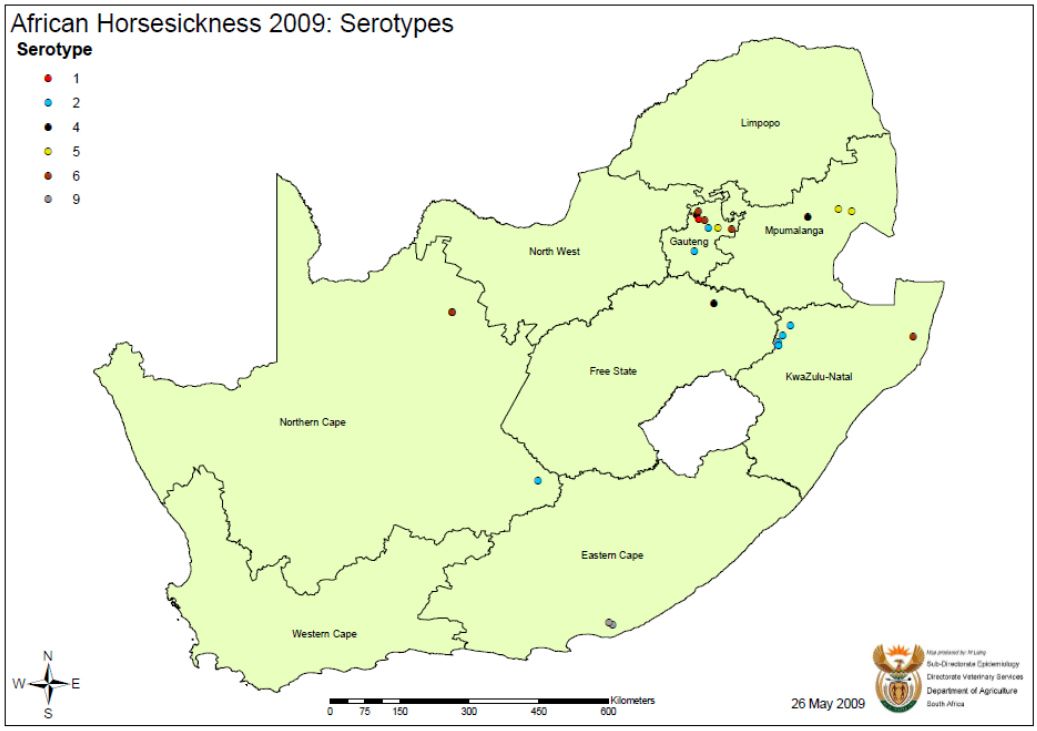

Figure 2.4: Serotype distribution in South Africa for the 2009 AHS season (DAFF, 2009).

2.4 Epidemiology

2.4.1 Epizootic Events

The distribution of AHS is limited to geographical regions that favour the biology of the *Culicoides* spp. midge (Mellor, 1993). AHS is endemic to sub-Saharan Africa, including both the tropical and sub-tropical regions. AHS occurs from Senegal in the west to Kenya and Somalia in the east and south down to South Africa. The disease has been reported in North Africa, but it is generally constrained to the south of the Sahara due to the desert's large expanse that acts as a protective barrier. AHS has not been reported in Madagascar (Mellor, 1993; Mellor & Hamblin, 2004; Guthrie, 2008).

Outside of Africa, AHS has occurred regularly in the Middle East. It was reported there in 1930, 1944 and 1959-1961. In 1965, AHS occurred in the northern-most African countries and crossed into Spain in 1966, but was quickly suppressed through a mass vaccination and slaughter policy (Lord *et al.*, 1997b). The virus usually fails to persist outside of Africa due to the climatic conditions experienced in winter (Mellor & Hamblin, 2004). The Iberian peninsula was again afflicted in 1987-1990 following the importation of AHSV serotype 4-infected zebra (Coetzer & Erasmus, 1994). The disease

disappeared with the arrival of winter and that was considered the end of it. However, the same serotype surfaced again in 1988 and every year until 1991, spreading to Portugal and Morocco. The apparent ability of the virus to overwinter outside Africa caused widespread concern and led to a wave of AHS research in Europe, which the literature shows, but the research has sadly dwindled. It has been elucidated that the virus was able to overwinter on the Iberian Peninsula as the winters are mild enough and the *Culicoides* midge, a competent vector, native to the region and active throughout the year, exists (Mellor & Hamblin, 2004). However, with an increase in the international movement of horses, the possibility of an outbreak occurring in countries previously free of the disease has increased greatly (Archer, 1974; Anonymous, 2008).

AHSV is only able to survive in a long-term fashion through continuous and uninterrupted cycles of transmission between vertebrate and invertebrate hosts. Any 'vector-free' period cannot be longer than the duration of viraemia. In sub-Saharan Africa, no such 'vector-free' periods exist and the virus is able to survive from season to season, year to year. The further north and south one goes, the climatic conditions become less conducive to the survival of the vectors and the cycle is broken, resulting in brief epizootics of the disease when specific environmental conditions are met, such as in the Iberian peninsular and Morocco (Mellor, 1994).

2.4.2 Endemic Events - South Africa

2.4.2.1 Geographical Distribution

In South Africa, AHS occurs every year, mainly east of the Karoo and Kalahari deserts (Figure 2.5 and 2.6) (AHS-Trust, 2008), but depends largely on the immune barrier (vaccinated population) and the timing within the season with regard to rainfall and ambient temperature (Gerdes, 2006). The disease historically and commonly appears to originate in the north-eastern parts of the country and move southward. Indeed, Lord *et al*. (2002) describe the force of infection as being the strongest in the north-east, declining in a south westerly direction. However, the 300,000 doses of vaccine that are sold by OBP per year appear to have slowed this southward progression somewhat and formed what could be termed an 'immune barrier' (Guthrie, 2008). Theiler (1921) reported the absence of the disease from large parts of the Free State and Lesotho. AHS predominates in the warm coastal areas or low-lying, moist, inland areas.

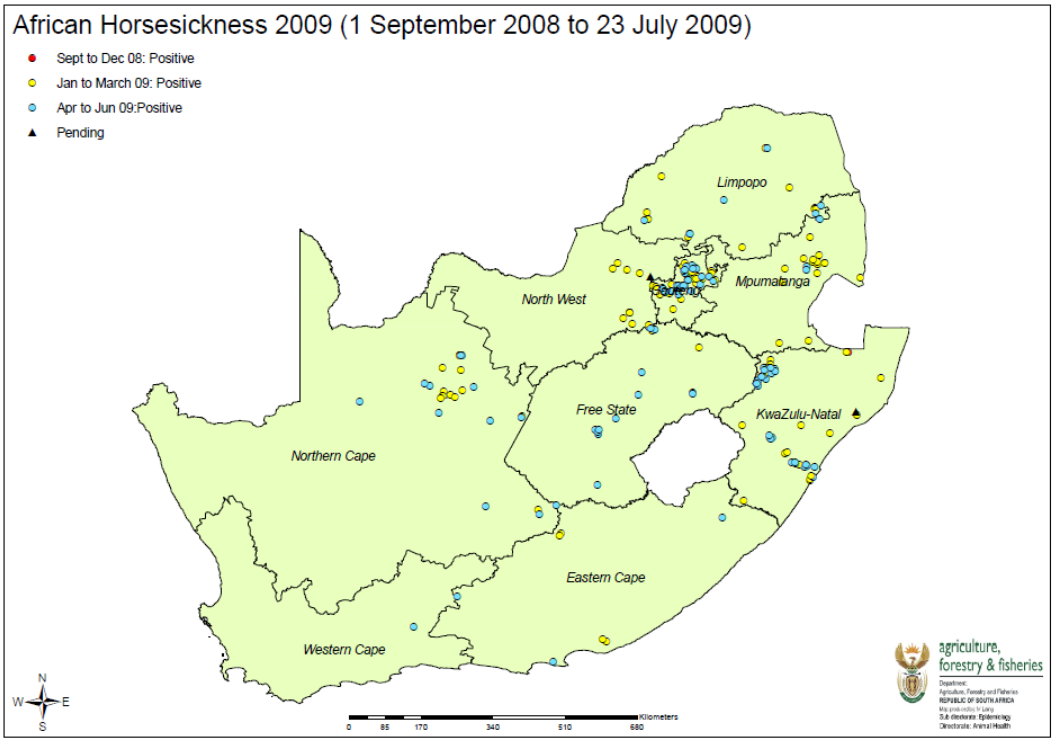

Figure 2.5: Confirmed outbreaks of African horse sickness in South Africa for the 2008/2009 season according to the National Department of Agriculture (DAFF, 2009).

Figure 2.6: Confirmed outbreaks of African horse sickness in South Africa for the 2008/2009 season according to the African horse sickness Trust (AHS-Trust, 2008)

Theiler (1921) also reported that the disease occurs predominantly in and around lakes, pans, vleis and rivers of the South Eastern and Eastern coastal belt. Heavy, early summer rainfall, followed by a drier period appears to favour the development of epidemics.

2.4.2.2 Seasonal Distribution

The disease seems to have both a seasonal (Figure 2.7) and cyclical nature. It is seasonal as it peaks during the late summer/autumn and cyclical as it has been proposed that the major outbreaks have occurred following El Niño warm-phase events, which occur approximately every 20 years (Theiler, 1921; Coetzee, 2000; OIE, 2004a). In general, AHS first emerges during February with a peak in cases during March and April. Following the first frosts in May/June, the disease appears to be arrested (Coetzer & Erasmus, 1994; Guthrie, 2008). However, data collected by the AHS Trust shows that cases are being reported from early December to May or June (AHS-Trust, 2008). Generally, the severity of the annual outbreak is assumed to be largely due to a complex combination of El Niño patterns, especially in a water-scarce country such as South Africa (Gerdes, 2006). Early and high precipitation summer rains tend to lend themselves towards an epizootic (Guthrie, 2008).

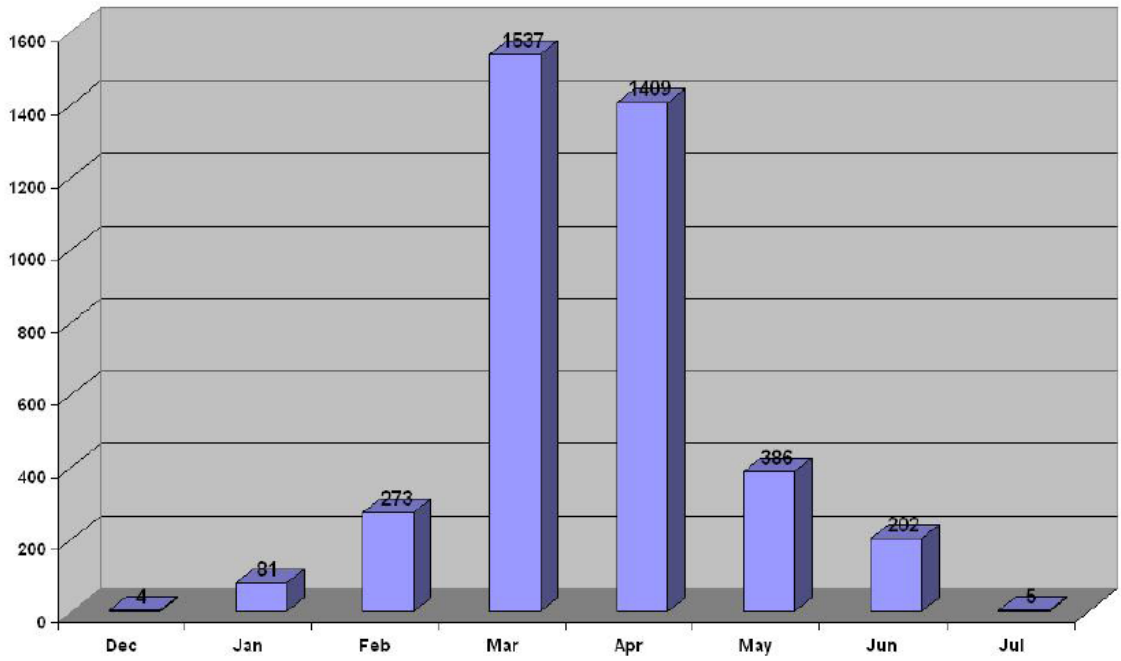

Figure 2.7: Number of cases reported between 2000 and 2006 showing the seasonal nature of African horse sickness. The number of cases reported between September and November are zero or negligible (Parker, 2008).

2.4.3 Seroprevalence

Usually, during an outbreak of AHS, one serotype may dominate, but it does not exclude other serotypes (Guthrie, 2008). Moreover, serotypes are seldom detected in the same area in successive years. Serotypes 1-8 are mostly responsible for outbreaks in South Africa, while serotypes 3, 4 and predominantly 9 have been recorded in outbreaks outside of Africa (Mellor & Hamblin, 2004). Serotypes 1-8 have been found to have a higher mortality (90-95%) than serotype 9 (70%) (Coetzer & Erasmus, 1994). All nine serotypes have been detected in eastern and southern Africa, with serotype nine being more dominant in the north west regions of sub-Saharan Africa (Guthrie, 2008). In 2006, AHS serotype 2 was found to be responsible for the outbreak in Nigeria that subsequently spread to Senegal. This was the first isolation of serotype 2 in the Northern Hemisphere. Reasons for this incursion range from lax border controls in West Africa to the spread of the vector due to global warming (Fasina, 2008; Fasina *et al.*, 2008).

2.4.4 Simulation Modelling

Simulation modelling of epidemics can facilitate understanding of the factors involved in the causation and duration of epidemics, particularly when they occur in non-endemic regions. Lord *et al.* (1996b) suggested that midge population size, the recovery rate in horses and the time of year that the virus is introduced to an area, were found to be the most significant factors to contribute to the establishment of an epidemic. The size of the epidemic was found to be influenced by the inter-bloodmeal interval of the midge, the mortality and recovery rates of the infected horses, midge population size and transmission rates.

2.4.5 Host Range

The range of hosts for AHS is confined mainly to equine species. The most susceptible are horses (70-95% mortality) followed by mules (50-70% mortality). Donkeys and zebras are the most resistant and seemingly remain subclinical for AHS (Theiler, 1921; Coetzer & Erasmus, 1994). The high mortality of horses and mules indicates that they are most likely accidental hosts (Erasmus, 2004). Varying individual responses to the same virus has been recorded (Theiler, 1921). Native horse populations in AHS

endemic/enzootic regions descended from herds from 2000 BC may have acquired resistance to AHS equal to a zebra or donkey (Coetzer & Erasmus, 1994). This may support the anecdotal evidence of subclinically infected horses infected with the virus and acting as reservoirs.

Another host for AHS is the domestic dog (Coetzer & Erasmus, 1994). However, the dog only becomes viraemic after the ingestion of infected meat or experimental infection (Mellor & Hamblin, 2004). They are thus not considered to play any role in the transmission of AHS (Coetzer & Erasmus, 1994; Braverman & Chizov-Ginzburg, 1996; Mellor & Hamblin, 2004). Other African carnivores that have been tested positive for AHS antibodies are the spotted hyena, lion, cheetah, African wild dog, jackal and genet (Alexander *et al.*, 1995). AHS antibodies have not been found in any wild or domestic ruminants save for the camel (Mellor & Hamblin, 2004). Pigs, cats and monkeys are resistant to infection (Coetzer & Erasmus, 1994).

Since the beginning of the 20th Century, the number of outbreaks occurring throughout South Africa appears to have been declining. This pattern coincides with a reducing number of free ranging zebra across South Africa because of hunting. However, the proliferation of game parks (and restocking of zebra) in certain areas is cause for concern. It is possible that at some undefined population density, a permanent host population may, therefore, become established in localised regions (Mellor & Hamblin, 2004).

The OIE reports that there is no evidence that humans could become infected with AHSV. However, it has been described previously that certain neurotropic vaccine strains may cause encephalitis and retinitis of the eyes in humans following aerosol infection during vaccine production (OIE, 2004a).

2.4.6 Reservoirs

“The continued circulation of an insect-transmitted virus depends on the availability of susceptible hosts and competent vectors in sufficient numbers” (Barnard, 1993). Zebra (*Equus burchelli*) are considered to be the natural vertebrate host and reservoir for the disease and are instrumental in the persistence of the disease in Africa (Bigalke, 1994). Experimental infection of zebra, and the identification of anti-AHSV antibodies in free-

living zebra is strongly suggestive of the reservoir role that zebras might fulfil in the persistence of AHSV (Barnard, 1993). Furthermore, the viraemic state in zebra may extend to up to 40 days (and remain subclinical), whereas in horses it is less than 7 days (Barnard *et al.*, 1994; Meiswinkel & Paweska, 2003; Maree & Paweska, 2005). Zebra are known to have existed on the Highveld and southern savannah woodland in South Africa for perhaps millions of years and to have adapted to the infectious agents of the environment and so may act as carriers and maintenance hosts (Bigalke, 1994). The reservoir host theory has yet to be conclusively proven (Erasmus, 2004; OIE, 2007). The literature that exists concerning zebra makes this assumption without setting out to prove or disprove the assumption (Meyer, 2007). Although it is commonly accepted that viral transmission is halted in winter, it may still continue at a lower rate in warmer, low-lying, tropical regions. In South Africa, this description would apply to the Kruger National Park. Coupled with the large numbers of zebras in the park, this may indeed constitute the reservoir host system needed for the virus to overwinter (Meiswinkel *et al.*, 1994). In a similar fashion, donkeys may perform the same role in other parts of the country where the population is high relative to the number of horses (Guthrie, 2008). The lack of zebra in areas outside Africa where outbreaks have occurred and not persisted, is indicative of the fact that the horse is not a long-term reservoir for AHS. However, serotype 9 persists in areas of West Africa where zebra do not naturally occur (Mellor & Hamblin, 2004). In 2007, it was reported that the Equine Research Centre at the University of Pretoria was looking into the role of zebras in AHS epidemiology (Anonymous, 2007d). It has been accepted that subclinically infected zebras imported into Spain were responsible for the Iberian outbreak in the late 1980's (Lubroth, 1988; Mellor & Hamblin, 2004). Dogs are unlikely to act as reservoirs (Braverman & Chizov-Ginzburg, 1996).

2.4.7 Vectors

The vectors for AHS are the crepuscular biting midges of the *Culicoides* genus (Figure 2.8), which transmit the virus by biological means (Coetzer & Erasmus, 1994; Mellor, 1994; OIE, 2007). They are distributed virtually worldwide (Meiswinkel *et al.*, 1994). Generally, midges are only dispersed a few kilometres from their breeding sites, but it has been suggested that midges may be carried by wind for hundreds of kilometres (Mellor, 1994).

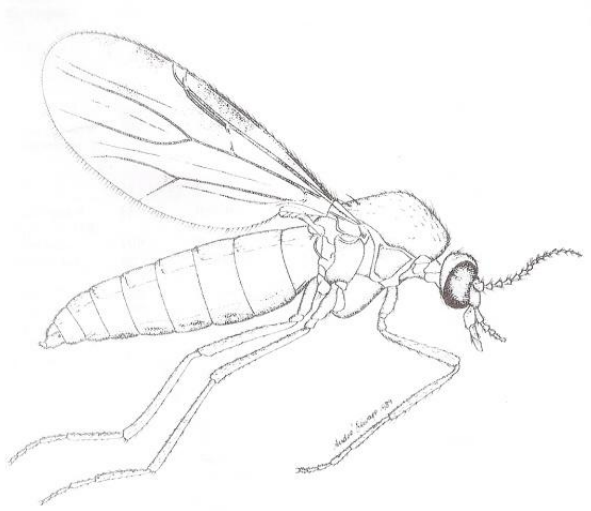

Figure 2.8: *Culicoides* spp. lateral view (Meiswinkel *et al.*, 1994)

The similarities between malaria and 'Horsesickness' led Theiler (1921) to believe that the vector was a blood-sucking insect. Various arthropod vectors have been considered and studied, chief among them being mosquitoes, ticks and *Culicoides* spp. (Wetzel *et al.*, 1970; Mellor, 1994), but only *Culicoides* spp. have been found to play any significant role in the spread of AHS (Mellor, 1993; Coetzer & Erasmus, 1994). In 1944, it was shown that wild caught *Culicoides* midges were infected with AHSV (du Toit, 1944). This was later confirmed by Mellor *et al.* (1975) and Boorman *et al.* (1975) who also showed that the transmission of the virus was possible after 7-10 days incubation at 26°C. The majority of experimental epizootiological evidence overwhelmingly suggests that the *Culicoides* species are the primary vectors of AHSV (Mellor, 1994). According to the OIE, certain mosquito and tick genera may constitute an occasional mode of transmission (OIE, 2007).

Culicoides imicola is found throughout sub-Saharan Africa, the Mediterranean Basin and South East Asia (Meiswinkel *et al.*, 1994; Mellor, 1994). In sub-Saharan Africa, *C. imicola* has been accepted as the primary vector of AHSV (Mellor & Hamblin, 2004). In 1982, it was found in Spain and has since been indentified throughout the Mediterranean and Southern Europe, even as far north as Switzerland (Goffredo & Meiswinkel, 2004). The occurrence of *C. imicola* across these regions may either be indicative of the improved and more intensive sampling strategies following the bluetongue epizootic (1998-2003), or that their range has moved further north due to global warming. Mellor and Hamblin (2004) suggest that it may be the latter. *Culicoides* samples taken in 1983 contained no *C. imicola.* Samples taken from 1999-2003 from

similar locations reveal that *C. imicola* is 'widespread and abundant'. Mellor and Hamblin (2004) further suggest that the presence of a viable vector for AHS (as in the Bluetongue outbreaks between 1998 and 2003) in these regions renders these areas particularly vulnerable to an AHS epizootic.

However, new research continues to surface about the role of the other species in the genus. As early as 1975, an American species, *C. sonorensis (=variipennis)* was shown to transmit the AHS virus (Boorman *et al.*, 1975; Mellor *et al.*, 1975). This also renders the North American continent susceptible to AHS outbreaks (Mellor, 2000). More recently, *C. bolitinos* was implicated in the transmission of AHS as a potential field vector. *C. bolitinos* is distributed widely in the cooler highland areas of South Africa where *C. imicola* is rare (Venter *et al.*, 2000). Isolations from *C. obsoletus* and *C. pulicaris* catches during the Spanish outbreak of AHS indicated that they may also transmit AHSV (Mellor *et al.*, 1990). Considering that *C. obsoletus* and *C. pulicaris* are the most common midge species across northern and western Europe, an AHS outbreak may not necessarily be confined to southern Europe. An additional *Culicoides* species that has been found to transmit the virus is *C. nubeculosus* that exists across Europe (Mellor et al., 1975).

The distribution of *C. imicola*, geographically and seasonally, is dependent on a range of environmental factors. Temperature is probably the most influential extrinsic factor affecting the transmission, infectivity and virogenesis of AHSV in *Culicoides* vectors and the survival of the midges themselves (Mellor, 1994; Wellby *et al.*, 1996; Mellor, 2000). The optimum temperature range for adult *Culicoides* spp. activity is 12.5 – 29°C. An increase in ambient temperature results in increased infection rates, faster virogenesis and earlier transmission. Survival rates of the midges, however, decrease. On the other hand, as temperature decreases, the opposite is true (Mellor, 2000) (Figure 2.9). The effect of temperature on different serotypes is not known.

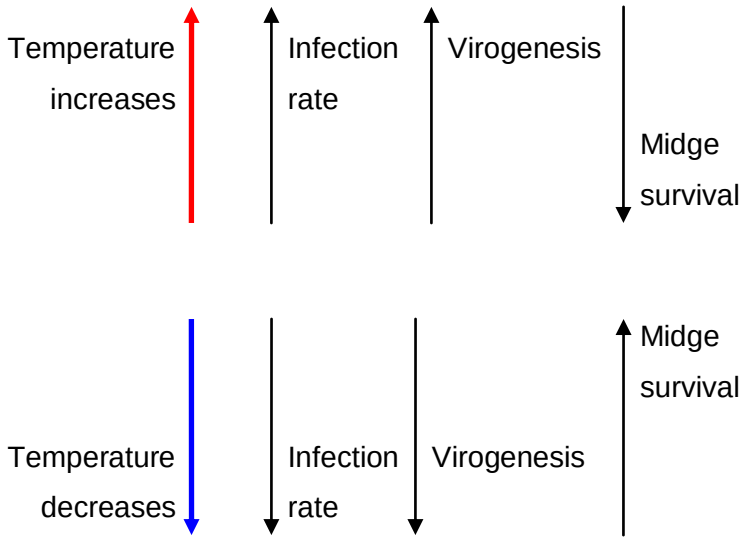

Figure 2.9: Different effects of ambient temperature on AHSV infection rates, virogenesis and midge survival rates (after Mellor, 2000; Mellor & Hamblin, 2004).

The infection rate falls to zero when ambient temperatures fall below 15°C. It appears, however, that virions persist in the midge at low temperature and when temperatures rise again, virogenesis commences (Wellby *et al.*, 1996; Mellor, 2000). It has also been shown in other studies (Sellers & Mellor, 1993; Mellor, 2000) that midges may be active at temperatures as low as 3°C. However, the virus cannot replicate at these temperatures. These findings suggest that even if there were to be an incursion into northern Europe by AHSV, the midge populations would increasingly lose the ability to transmit the virus the further north it extends. Transmission would only be possible over the short summer months and be arrested in winter. A caveat exists, however, that at these low temperatures, midge survival increases dramatically, to 90 days in some cases, in an inactive state. Should the local climate have a winter period (below 15°C average) of less than 90 days, it would be possible for the midge to carry over latent virus from the previous summer and begin a new cycle (Mellor, 2000).

Mellor & Hamblin (2004) view the above phenomenon as a possible over-wintering mechanism where vertebrate reservoirs are not available. However, Rawlings (personal communication in Mellor, 1994) suggested that an 'accumulation of cold stress' might be a major fact in *Culicoides* mortality, as opposed to a single cold event. This theory discredits the commonly accepted fact that the first frost of the season totally arrests midge activity for the duration of the winter (Mellor, 1994). When considering possible overwintering mechanisms, it is important to note that viruses

appear unable to pass trans-ovarially in midges (Meiswinkel *et al.*, 1994). However, with the global warming phenomenon, the above factors that combine to prevent AHS over-wintering in Europe successfully now may not be enough to prevent AHS from making a permanent and resident incursion (Wittmann & Baylis, 2000; Wittmann *et al.*, 2001)

2.4.7.1 Viral cycle in Culicoides *spp.*

When a female *Culicoides* spp. takes a blood meal from a viraemic equine, the virus is deposited in the lumen of the mid-gut. The virus infects and replicates in these luminal cells from where the virus is released into the haemocoel and infects the secondary targets, such as the salivary glands. The virus continues to replicate in the salivary gland for the life of the midge. When the female takes another blood meal, the virus passes from the salivary glands into the blood stream of the animal (Mellor, 1993).

2.5 Pathogenesis

The severity of the disease depends largely on the virulence of the virus, the infective dose (related to the number of infected midges that bite the animal) and the susceptibility or immunological status of the animal. The factors and molecular basis that determine the virulence and pathogenesis characteristics of AHSV are not well known, although it is suspected that they lie with the proteins VP2, VP5 and NS3 and are multifaceted (Huismans *et al.*, 2004). Clinical signs develop as a result of the damage to the endothelial cells in blood vessels and reduced function of the circulatory and respiratory systems (Mellor, 1993).

Initial viral multiplication occurs in the regional lymph nodes resulting in a 'primary viraemia' that disseminates the virus throughout the body via the blood and leads to the infection of target organs such as the lungs, spleen and lymphoid tissues. From the infected organs, a 'secondary viraemia' occurs, of varying titre and duration, depending on the host species (Coetzer & Erasmus, 1994). In horses, the maximum titre is usually 10^5 $TCID_{50}$/mL with the viraemia lasting 4-8 days and paralleling the febrile reaction, although it may vary between 2-21 days (Erasmus, 2004). In donkeys and zebras, the viraemia is considerably lower and may last for up to 28 or 40 days depending on the study (Hamblin *et al.*, 1998; Erasmus, 2004; OIE, 2004a). It has been found that in zebras, viraemia may co-exist with circulating antibodies (Coetzer & Erasmus, 1994).

In experimental infections, the incubation period lasts 5-7 days with a minimum of 2 days and a maximum of 10. This has been found to be dependent on the dose and virulence of the virus(Guthrie, 2008). The effect of infecting serotype has not been determined.

Immediately after infection, AHSV rapidly accumulates in the spleen, lungs, caecum, pharynx, choroids plexus and most lymph nodes. AHSV subsequently moves to other highly vascularised organs (Mellor & Hamblin, 2004). Only trace amounts are found in secretions (Erasmus, 2004). AHSV was reported by Theiler in 1921 to be closely associated with erythrocytes. More correctly, it has since been found to be associated with the cellular fraction, i.e. the erythrocytes and lymphocytes, with very little in the plasma. This phenomenon may be similar to BTV infections where the virus is sequestered in the cell membrane of infected erythrocytes (Mellor & Hamblin, 2004). The damage inflicted by AHSV on mammalian cells is likely due to the damaging exit mechanisms from the cell that the virus employs (Mellor, 2000). In a 1999 study on the AHS virus and its effects on capillaries, it was found that the virus was most common in the myocardial vessels and least common in the lung, while endothelial cell infection was rare in the spleen and liver (Gomez-Villamandos *et al.*, 1999).

Since 1921, when Theiler first described them, the four ‘forms’ of AHS have been used to categorise the disease. In ascending order of severity, these are the horsesickness fever, the subacute/oedematous (cardiac or ‘dikkop’) form, the mixed form and the peracute (pulmonary or ‘dunkop’) form. It has since become apparent that most infections are of the ‘mixed’ form (Coetzer & Erasmus, 1994; Mellor & Hamblin, 2004).

Horse sickness fever is the mildest form of the disease and usually involves a mild fever and slight oedema of the supraorbital fossae. Mortality is rare and occurs in animals with some immunity. It is the only form seen in zebras and donkeys. In the *cardiac form*, a fever may persist for a few weeks and there is significant subcutaneous oedema, particularly of the supraorbital fossae, but none in the lower limbs. Petechial and ecchymotic haemorrhaging (of the eyes and of the tongue respectively) may be present and mortality may exceed 50%. Colic is often a feature. The most severe form is the *pulmonary form*, where there is a mortality rate of 95%. It develops very rapidly with a fever of up to 41˚C followed by signs of respiratory distress. Foam exudes from the nostrils as the animal dies. The pulmonary and cardiac forms are often found to

afflict the animals simultaneously. This *mixed form* is the most common with a mortality rate of 70%. Animals usually die within 3-6 days (Coetzer & Erasmus, 1994; Mellor & Hamblin, 2004).

Laegreid *et al.* (1993) observed that the infecting serotype determines to some extent the form of the disease that is exhibited in the animal. Sailleau *et al.* (1997) added to this observation when horses experimentally infected with serotype 4 developed the pulmonary form and those infected with serotype 9 developed the cardiac form. However, the relationship between each serotype and the form of the disease or mortality rate remain undetermined.

2.6 Control and Prevention

There is no specific treatment for African horse sickness (Mellor & Hamblin, 2004). Theiler (1921) reported that the best treatment might be to leave the horse alone. Combating AHS therefore moves towards prevention of an infected insect bite and control by 'rendering the animals immune' (Theiler, 1921). Foremost among these measures are introducing good husbandry practices, controlling the *Culicoides* midge vector and vaccination (Meiswinkel *et al.*, 1994). The large number of horses that transverse international boundaries in today's modern equine industry constitute an increasing concern due to the threat of various viral diseases of equines (MacLachlan *et al.*, 2007). Vaccination is seen as key to protecting the global nature of the equine industry.

2.6.1 Control

Since *Culicoides* midges are crepuscular (Wittmann & Baylis, 2000), husbandry measures include housing animals from before dusk to after dawn and preventing access of the midge to the building (Wittmann *et al.*, 2001; Erasmus, 2004). The application of insecticides to the animals' coats may deter the midge from biting. These measures aim to limit the amount of time the animals can be exposed to the vector ('bite load'). Even before the nature of the vector species was determined, the above measures were found to be highly effective at preventing infections (Theiler, 1921) and are still effective today. Controlling vector populations aims to reduce the number of potential bites that susceptible animals receive. Eradicating the midge population entirely is not possible, nor is it wise, in an ecological sense. Controlling the population

includes altering their habitat, adultciding, larvaciding and repellents (Jenkins, 2008; Simpkin, 2008). Additionally, in the case of outbreaks, movement restrictions may be put in place and slaughter policies introduced in the case of currently AHS-free countries (Portas *et al.*, 1999; Mellor & Hamblin, 2004).

2.6.2 Vaccination

The most practical approach and primary means to the prevention of viral diseases is vaccination (OIE, 2004b; MacLachlan *et al.*, 2007). The distribution of the nine AHS serotypes is throughout South Africa, although they may differ temporally (Coetzer & Erasmus, 1994). For this reason, a polyvalent, attenuated vaccine was developed by OBP.

There have been a number of different vaccines developed for AHS over the last century. The initial approach was to inoculate horses with virulent virus and immune sera (MacLachlan *et al.*, 2007). Early vaccines were produced in the 1930s by passaging the virus approximately 100 times intracerebrally in suckling mouse brain and were called 'horsesickness neurotropic mouse brain vaccines'. Despite the good protection that these vaccines provided, they occasionally resulted in serious side-effects with some horses dying of encephalitis (Nobel & Neumann, 1961; Pavri & Anderson, 1963) and proved to be infectious to humans (MacLachlan *et al.*, 2007). These problems were solved in the 1960s by passaging in cell cultures instead (Mirchamsy & Taslimi, 1964; Mirchamsy & Taslimi, 1968). Also in the 1960s live-virus and killed-virus vaccines were investigated (Ozawa *et al.*, 1965; Ozawa & Bahrami, 1966). In 1974, however, these vaccines began to be replaced with plaque variants of AHSV grown in Vero cells (Coetzer & Erasmus, 1994; OIE, 2004a).

The current vaccine produced by OBP remains the best and most practical means of protection against AHS (Figure 2.10). However, the vaccine cannot be relied upon to give full protection to the animal. The individual's response may vary, there may be some interference between the serotypes in the polyvalent vaccine or over attenuation of some of the vaccine strains, leading to weakly immunogenic vaccine strains (Coetzer & Erasmus, 1994). Historically, however, the development of the polyvalent vaccine has significantly reduced the losses associated with AHS (Guthrie, 2008). In a modelling simulation, it was determined that 50% of epidemics may be avoided if 75%

of horses and donkeys or 90% of horses only were vaccinated before the introduction of the virus. Importantly, donkeys needed to be included in vaccination programs to reduce overall losses. However, this model was based on a two host, one vector system in Spain (Lord *et al.*, 2002). This is far from the situation in South Africa, but gives an indication of the importance of vaccination.

Figure 2.10: African horse sickness vaccine manufactured by Onderstepoort Biological Products (http://www.obpvaccines.co.za/prods/54.htm)

Annual immunisation is recommended for September each year, before the peak AHS season, with yearlings normally receiving two vaccinations in the first year (Coetzer & Erasmus, 1994; Anthony *et al.*, 2004), with the timing of these vaccinations having been found to be of crucial significance (Crow, 2005). OBP manufactures two quadrivalent vaccines containing live, attenuated strains. The seed virus is selected from genetically stable macroplaques from Vero cells (OIE, 2004a). The first vaccine contains serotypes 1, 3 and 4 (AHS1), while the second vaccine contains 2, 6, 7 and 8 (AHS2). The vaccines must be administered at least three weeks apart. Serotype 9 is not included in the vaccine since it is very rare in South Africa and serotype 6 affords sufficient cross-protection. Serotype 5 was removed from the vaccine in 1993 due to reports of severe reactions in workers and deaths in horses and is cross protected by serotype 8 (Mellor & Hamblin, 2004; MacLachlan *et al.*, 2007). Repeated vaccinations over time are believed to assist the animal in gaining greater immunity to the serotypes contained in the vaccines (Coetzer & Erasmus, 1994). It is interesting to note, that in the last outbreak on the Iberian Peninsula from 1987 to 1991, a polyvalent vaccine was initially used. It has been claimed by some workers, although unpublished, that subsequent to these polyvalent vaccinations, different serotypes began appearing in animals that were not the initial serotype 4. This suggests that the polyvalent vaccine

produced a viraemia in the animals vaccinated with it and it was then transmitted by the resident vectors. This has, however, yet to be substantiated (Mellor & Hamblin, 2004).

Some anecdotal comments suggest that the vaccine itself may be responsible for inducing a fatal viraemia, particularly in younger horses (MacLachlan *et al.*, 2007). There is little literature to back up this claim and the vaccines used to control the last epizootic in Spain have 'never been known to revert to virulence' (Mellor, 1993). 'Vaccine-related deaths' during the Iberian outbreak of 1987-1991 were later said to be due to the vaccination of already infected horses or that, due to a lack of education, the horses were worked after vaccination (Portas *et al.*, 1999). A serology assay has been developed that could be used to differentiate a vaccine viraemia versus a natural viraemia (Laviada *et al.*, 1995). However, this assay requires specific reagents and is unlikely to find widespread acceptance. In 2008, a study was conducted to determine whether the vaccine manufactured by OBP could induce clinical symptoms. The study concluded that "assumptions of virulence or reversion to virulence of vaccine re-assortments post-vaccination in horses could not be substantiated" (von Teichman & Smit, 2008).

It would, however, be advantageous to conclusively trace the origin of the virus in an infected horse, be it wild type or vaccine strain. Vaccine and wild-type strains of the avian pathogen *Mycoplasma gallisepticum* have successfully been differentiated with standard PCR techniques (Evans & Leigh, 2008). More importantly, field and vaccine strains of the bluetongue virus were differentiated rapidly using real-time RT-PCR following reports of vaccine virulence in European outbreaks (Elia *et al.*, 2008).

Inactivated vaccines are an alternative to live, attenuated ones. They are advantageous in that they do not contain a potentially dangerous live agent. However, they are expensive to produce and require multiple inoculations. Complete vaccine inactivation may also be difficult. No such vaccines currently exist, although they have been developed and successfully used in the past (Mirchamsy & Taslimi, 1964; Mellor & Hamblin, 2004).

Howell (1962) recognised the advantages of monovalent vaccines over polyvalent vaccines, especially when outbreaks occurred in non-endemic regions. Monovalent vaccine production is simplified and expedient and allows for the reduction of economic

losses in an outbreak. Inactivated monovalent vaccines are used extensively in West Africa, where serotype 9 is the dominant circulating serotype (National Laboratory, Senegal) (Mellor & Hamblin, 2004). Whenever AHSV has appeared outside Africa, monovalent vaccines have successfully been used for the particular serotype involved. In the Spanish outbreak of 1987-1991, an inactivated monovalent serotype 4 vaccine was produced from the attenuated vaccine strain (OIE, 2004a).

Although popular and efficacious, live attenuated and inactivated vaccines are perceived to have many flaws. As far as AHS-free countries are concerned, the use of a live, attenuated vaccine as a preventative measure against an outbreak is equal to declaring that the AHS virus itself was present (Portas *et al.*, 1999). Recombinant vaccines represent a modern alternative, but few have reached a commercial phase (MacLachlan *et al.*, 2007). A considerable amount of research has been done on sub-unit vaccines, albeit for BTV (Savini *et al.*, 2007). Subunit vaccines have also been tested successfully with AHSV, although they are no longer commercially available. VP2, VP5 and VP7 of serotype 4 were expressed in baculovirus expression systems and used to immunise horses (Martinez-Torrecuadrada *et al.*, 1996). A complete protective immune response was achieved, although only VP2 was soluble and produced neutralising antibodies. Recombinant VP2 from serotypes 3, 4, 5 and 9 have since also been used to successfully immunise horses (Bentley *et al.*, 2000; Martinez-Torrecuadrada *et al.*, 2001; van Niekerk *et al.*, 2001; Scanlen *et al.*, 2002).

Mellor and Hamblin (2004) recognise the success of the live, attenuated, polyvalent vaccine in endemic situations such as South Africa, but the authors have concerns regarding its use elsewhere, in epidemic situations:

1. There is no AHSV vaccine licensed for manufacture outside of Africa at present.
2. The vaccines produced by OBP are, as mentioned above, only available in two polyvalent live attenuated forms. In the case of an epidemic requiring emergency vaccination, there will be a delay in the implementation of a suitable vaccination program while a monovalent or different form of the polyvalent vaccine is manufactured.
3. The live nature of the current OBP vaccine renders it unsuitable for vaccination of pregnant mares due to possible teratogenic effects.

4. The vaccine strains used in South Africa are of South African origin. Using the vaccine outside of sub-Saharan Africa, could introduce a different virus type or strain into the eco-system.
5. It has also been suggested that since the vaccine is live, re-assortment of genome segments may occur between the vaccine strains and wild-type viruses. In a worst-case scenario, this may result in 'new' strains of the virus that may be more virulent with unique antigenic properties.
6. Using a live virus may result in a vaccine virus viraemia in some vaccinated equids. However, this has yet to be conclusively studied.
7. It is unknown whether *Culicoides* spp. would be able to transmit vaccine strains and facilitate re-assortment.

The concerns expressed above are those of Mellor and Hamblin (2004) and are echoed by Guthrie *et al.* (2009). In addition, the dangers surrounding live virus vaccines were already being discussed in the 1960s (Mirchamsy & Taslimi, 1968).

In response to a growing call for a modern vaccine candidate, Guthrie *et al.* (2009) have developed a recombinant canarypox-vectored monovalent vaccine for serotype 4. The genes of the outer capsid proteins VP2 and VP5 of AHSV were cloned into a canarypox vector. The proteins were expressed through the canarypox vector and, when inoculated into horses, induced neutralising antibodies, while remaining avirulent. An identical method had been successfully used previously for bluetongue virus, West Nile virus and equine influenza virus. Vaccination of horses with this new vaccine prevented the horses from becoming viraemic after inoculation with live AHS virus and resulted in appropriate circulating antibodies. This vaccine represents a huge step forward for the successful prevention of AHS, but it is monovalent and the infecting serotype will have to be determined prior to immunisation with the vaccine. Unfortunately, however, the complete development of a veterinary vaccine, from proof of concept to marketing authorisation, will take many years and must overcome many obstacles (Heldens *et al.*, 2008).

The design of modern vaccines, with a tendency to be serotype specific, must be based on a sound understanding of the molecular biology and pathogenesis of each serotype within the horse (MacLachlan *et al.*, 2007). Serotyping assays are crucial to increase this required knowledge.

Of concern with any viral disease and its control by vaccination is the antigenic diversity that exists because of mutation, recombination or re-assortment between different strains. Although AHSV appears to be a relatively stable virus genetically, surveillance programs to monitor the circulating serotypes are important to keep ahead of the virus (Mumford, 2007). Rapid serotyping assays would have the potential to identify shifting genotypes during an outbreak and facilitate monitoring of its progress in real time.

Apart from the above-mentioned vaccines, virus-like particles (VLPs) have also been used to induce an immune response. VLPs are a virtual replica of the virion, without a genome – in essence, a protein shell without the ability to replicate and induce a viraemia. This takes into account the disadvantages and shortcomings of other vaccine types, such as a lack of immunogenicity (sometimes experienced with sub-unit and recombinant vaccines) and incomplete activation of some viruses in inactivated vaccines. It also cannot revert to virulence due to its lack of (or partial lack of) a genome. VLPs have been developed for BTV (Noad & Roy, 2003).

2.6.3 Antiviral Therapy and Prophylaxis

In many epizootics, emergency vaccination is far from ideal due to the time lag between immunisation and the immune response. Some form of antiviral treatment for a confirmed, perhaps serotyped, infection would be ideal. Evidence exists that suggests that foot and mouth disease viral infections may be treated, but other RNA viral infections will have to wait until the disease and the virus are better understood (Goris *et al.*, 2008). Treatment at the moment is merely symptomatic and supportive (Guthrie, 2008).

2.6.4 AHS in the Global Village and International Control

The increase in the international movement of horses for the racing and sport horse industries has made AHS a very important disease to researchers and animal disease control officials across the world (Sakamoto *et al.*, 2000). The movement of horses increases the risk of spreading infectious diseases (Archer, 1974). The United States horse industry was reported to be worth $1.75 billion in 1998 and employs seven

million people (Dvorak *et al.*, 2004). An outbreak in the United States would be very damaging and distressing. In recent years, the bluetongue virus has been detected in increasingly northern regions in Europe, historically free of bluetongue (Mullens *et al.*, 2004; Vellema, 2008). Presently, AHSV is endemic to sub-Saharan Africa, having occasionally spread into regions geographically associated with Africa's boundaries, rapidly and without warning. The distribution of AHSV is associated with the distribution of *Culicoides* spp. capable of transmitting the disease. *C. imicola* has expanded its range northwards through Europe because of climate change and this is the most probable answer to the Bluetongue outbreaks in Europe in recent years (Mellor & Hamblin, 2004; Guthrie *et al.*, 2009; Maclachlan *et al.*, 2009). Serious concerns have been expressed in a number of news articles published online as to the possibility of AHS spreading into Europe as bluetongue did. Horsetalk.co.nz reports that the arrival of AHS into Britain would be a 'death knell' for all forms of equine sport according to The Horse Trust (Anonymous, 2007b; Lesté-Lasserre, 2007). With the first case of bluetongue in Britain in the latter half of 2007, many consider the arrival of AHS inevitable (Anonymous, 2007a). Medreonet, a French Agricultural Research Centre for International Development (CIRAD) research unit has been formed to monitor three Orbiviruses, including AHS, since the emergence of bluetongue in Europe (Anonymous, 2007c). The importance of a rapid assay for diagnostics becomes exceptionally important should an outbreak of AHS occur in a country free of the disease and with an equine population 100% naive to the virus.

AHS is considered to be the most important disease of equines to be evaluated when moving horses across international borders (Guthrie, 2008). It was first discussed at an international conference in 1967 (Ditchfield & Thomas, 1967) and has since featured prominently in many related conferences. The OIE has defined a number of zones for the control of AHS globally. A 'free country' is a country where no confirmed infection has occurred for the last two years and where no horse has been vaccinated in the last year. In a country where the disease is endemic, a free zone may be declared as for a free country, and should preferably be delineated by substantial geographic boundaries with suitable animal movement controls (OIE, 2007).

In South Africa, after the European Commission Decision in 1997 (97/10/EC), exports were allowed to move directly out of the Cape Town Metropolitan area as long as the free zone was defined (Parker, 2008). The free zone is located in Cape Town with

appropriate buffer zones throughout the Western Cape (Figure 2.11). However, this has not been foolproof as AHS cases occurred in the controlled area in 1999, 2004 and 2006 (Parker, 2008). Export of horses from an infected country must take place through quarantine stations within the free zone (OIE, 2007). In South Africa, the Department of Agriculture has devised a control policy for AHS in line with EU and OIE regulations. These policies are laid out in the regulations pertaining to the Animal Diseases Act (Act No. 35 of 1984)[1]. Importantly, it states that all horses, mules and donkeys ***must*** be vaccinated annually and in the free and surveillance zones of the Western Cape, permission must be obtained from the relevant Director of Veterinary Services to vaccinate equines.

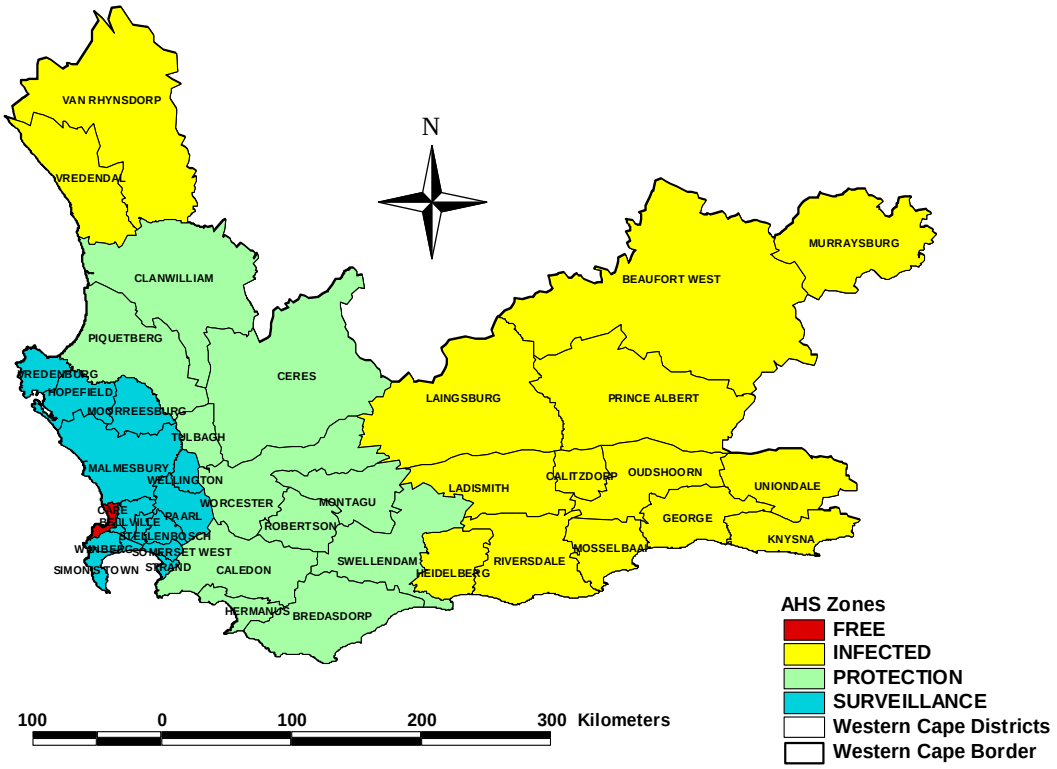

Figure 2.11: South African AHS Controlled Area devised in 2001 for the continued export of horses from South Africa (DoA, 2003).

Some of the more salient points with regard to the AHS Controlled Area include (DoA, 2003):

- Horses moving into the control zone must be vaccinated by a veterinarian or under supervision of the State Veterinarian and 60 days must have elapsed between vaccination and movement into the control zone.

[1] http://www.nda.agric.za/vetweb/Legislation/Gov%20Gaz%20-%20Act%2035%20of%201984%20-%20Part%201.pdf

- Appropriate Health Certificates and Movement Permits must be in order
- All movement of equines is prohibited into the Control Zone during the height of the season, i.e. March to June each year unless they are quarantined for three weeks in the Protection Zone.

In countries previously free of the disease, determining the serotype and producing a monovalent vaccine is of a priority, should there be an outbreak. A monovalent vaccine is the most successful control measure as it results in long-lasting immunity in most animals.

In countries/territories where an epizootic of AHS has occurred, the disease has successfully been eradicated and declared AHS free. In Morocco, which suffered an epizootic following the outbreak on the Iberian Peninsula in 1989, a number of steps were taken. Surveillance committees were set up, insect control was initiated at all ports of entry from Spain, equine imports were banned, a bank of monovalent vaccine was raised and rapid diagnostic assays were bought. Following the first case in Morocco, a vigorous vaccination program was started. More than 72% of the equine population at risk were vaccinated. Although only 17% of the diagnosed animals were reported to have died, all confirmed cases were destroyed. Subsequently, a vaccination program continued into 1994, with a reported coverage of 92%. Morocco remains free of AHS to this day (Benazzou *et al.*, 2006).

In some spheres of European and American society (where AHS is non-endemic) there is a concern that the AHS virus may be used as a biological weapon (Beck, 2003). This scenario, whether likely or not, would create the same environment that a naturally occurring outbreak would in a non-endemic region. In the author's opinion, rapidly serotyping and distributing the correct monovalent vaccine is of utmost importance.

After the 2004/2005 outbreak of African horse sickness in South Africa, a task team was formed by various role-players of the SA equine industry and it has subsequently evolved into the AHS Trust. As part of its mission, the Trust has initiated an extensive publicity campaign, is working with national authorities on reporting strategies and embarks on regular vaccination campaigns. As part of an ultimate goal, it seeks to improve on the current export protocol as well (AHS-Trust, 2008; Parker, 2008).

2.7 AHS in rural, subsistence communities

One of the major problems in South Africa with regard to the outbreaks is the large population of unvaccinated equids living in the rural parts of the country, away from the concentrated racing and sport horse communities (Gerdes, 2006). Many of these equids (mainly rural subsistence horses and donkeys) are used as traction animals or for farming in a variety of activities. A survey of animal traction in South Africa by Simalenga and Joubert (1997) defines animal traction as the use of animals (including donkeys, mules and horses) to assist farmers:

- in agriculture (e.g. ploughing, harrowing, planting, ridging, weeding, mowing and harvesting);
- in transport, for pulling carts and loads over a surface, logging and carrying loads (pack animals);
- in irrigation, for driving water-pumps and pulling water from wells;
- in the building industry, for assisting in earth moving for road works, for carrying bricks, etc.;
- to provide power for the operation of stationary implements such as threshing machines, grain mills and food processing machines.

It is estimated that over 100 million equines are still used and relied upon for draught and transport in subsistence agricultural communities around the world. Despite the enormous role that these equines play in their communities, little attention is given to health and welfare of these animals and veterinary authorities largely ignore these populations that may play an important role in endemic and non-endemic situations of AHS (El Idrissi & Lubroth, 2006).

In an earlier survey carried out in 1994, it was established that in the rural areas of the South Africa, 40 to 80 % of the smallholder farmers visited were using animal power for transport and cultivation. Simalenga and Joubert (1997) pointed out numerous benefits of using equids in animal traction, chief among them being their low cost and versatility compared to cattle. The result of the above is a large number of equids being used in rural areas for small-scale, subsistence farming (Segwagwe *et al.*, 2000), with most, if not close to all, animals being unvaccinated against AHS. When an outbreak occurs, these populations could provide a reservoir for the disease.

It has been recognised, particularly in eastern Africa, that community involvement in animal disease control provides an effective channel through which rural, subsistence communities can be reached. Vaccinations and diagnostics can be performed by community based health care workers with success (Catley & Leyland, 2001). With regard to AHS, communities that rely on horses, mules or donkeys should be targeted in order that a more effective national campaign to control AHS might enjoy more success.

2.8 Diagnostic Methods for African horse sickness

Our understanding of AHS depends largely on accurate diagnostics and the type of assay used. Field diagnosis is limited to clinical symptoms. The diagnosis of trans-boundary diseases, such as AHS becomes particularly important as the diagnosis will significantly effect the export/import status of that country or region (Rodriguez-Sanchez *et al.*, 2008b). Laboratory diagnosis of AHS is essential due to its notifiable status and should be confirmed by isolation or identification of the actual virus (OIE, 2004a). Usually this can be achieved through whole blood collected in the presence of an anti-coagulant or samples of the spleen, lung, lymph nodes or salivary glands (Mellor & Hamblin, 2004; OIE, 2004a). In turn, due to the multi-serotype nature of the virus, diagnostic assays may go further than a simple positive or negative result to serotype the virus.

2.8.1 Antibody Identification / Serological Tests

A number of diagnostic assays have been developed to detect group specific antibodies, mainly against VP7 (Segment 8) due to its highly conserved nature across all nine serotypes (Roy *et al.*, 1994). The main tests used locally and globally are complement fixation, agar gel immunodiffusion, immunofluorescence and enzyme-linked immunosorbent assays (ELISA) (Williams, 1987; Hamblin *et al.*, 1990; House *et al.*, 1990; Hamblin *et al.*, 1991; OIE, 2004a). To detect serotype specific antibodies, serum neutralisation tests (SNT) are available (Blackburn & Swanepoel, 1988; House *et al.*, 1990). SNTs are applied mainly in epidemiological surveillance and transmission studies (Mellor & Hamblin, 2004). Although VP7 may be desirable for serological detection due to its conserved nature, anti-VP7 antibodies are only formed 15 days after infection, which is highly unsuitable for rapid diagnosis. The earliest serological

markers to be detected are against VP5 followed by VP6 and NS3; VP6, however, appears to be well conserved across orbivirus species (Martinez-Torrecuadrada *et al.*, 1997)

The OIE prescribed test for international trade for AHS is the indirect ELISA. OIE Reference Sera exist in order to standardise the test and are held by the OIE Reference Laboratories. In South Africa, this laboratory is located at the Onderstepoort Veterinary Institute (OVI) (OIE, 2004a). Recombinant VP7 is the antigen of choice due to its stability, lack of infectivity and highly conserved nature (Roy *et al.*, 1994). Using VP7, the tests are also highly sensitive and specific (OIE, 2004a). An indirect ELISA using NS3 as the antigen was developed by Laviada *et al.* (1995) to distinguish between circulating antibodies produced because of vaccination or infection. However, inactivated monovalent vaccines were used. This does not apply to the polyvalent vaccines produced by OBP in South Africa.

Immunoblotting (Western Blotting) involves the separating of viral proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferring them to a nitrocellulose membrane. The nitrocellulose membrane is subsequently incubated in the test sera and then enzyme-conjugated immunoglobulins and positive bands are revealed using the appropriate substrate. When compared to positive and negative controls, two or more bands that correspond to bands on the positive control can be regarded as a positive result (OIE, 2004a).

Complement fixation (CF) is the second assay recognised by the OIE as a prescribed test for international trade. CF is being used less and less today because of the high sensitivity and specificity achieved with the ELISA tests. It is often used for the detection of group-specific antibodies to AHSV (OIE, 2004a).

Virus neutralisation is the OIE's choice for serotyping and the method is based on the work of Hazrati and Ozawa (1965; 1968) and House *et al.* (1990).

2.8.2 Antigen Identification / Identification of the Virus

Haemagglutination

One of the first methods used to identify viruses is the haemagglutination assay and uses erythrocytes of avian or mammalian origin to agglutinate with viral particles, giving a directly visualised diagnosis of the presence of a virus or not (Hierholzer & Suggs, 1969; Hierholzer *et al.*, 1969).

Viral isolation

The gold standard for identifying AHSV is viral isolation and this represents the only way of positively identifying an active infection (Koekemoer *et al.*, 2000). Virus isolation has been the traditional choice for the successful identification of orbivirus species (Eaton & White, 2004). Viral isolations can be achieved using baby hamster kidney cells (BHK21), African green monkey (Vero) or monkey kidney (MS) cells (Erasmus, 1963; Ozawa *et al.*, 1965; Ozawa & Bahrami, 1966; Mellor & Hamblin, 2004; OIE, 2004a). Hamster kidney cells were the first reported cells used for the tissue culture of AHSV (Erasmus, 1964). According to Mellor & Hamblin (2004), intracerebral inoculation of suckling mice (1-3 days old) is the preferred method today. If the mice develop neurologic symptoms within 3-15 days post-infection, the result is positive. A second passage must be performed using the initial mice's brains, homogenised and again inoculated intracerebrally to result in 100% infectivity in an incubation period of 2-5 days (Eaton & White, 2004; OIE, 2004a). Embryonated chicken eggs can also be used successfully (Erasmus, 1964; Mellor & Hamblin, 2004; Paweska, *Personal Communication*). It has, however, been reported that viral isolation may not be possible in less severe forms of the disease or strains that produce mild symptoms (Mellor & Hamblin, 2004). Transport and storage conditions also appear to affect the viability of the virus for isolation. For best results, inoculation of the cell cultures should happen immediately after collection of the virus. Viral isolation may also prove more difficult when the strain is less virulent (Sailleau *et al.*, 1997).

Enzyme-linked immunosorbent assay (ELISA)

An ELISA has also been developed that is very useful and rapid in identifying AHS viral particles in tissue samples from dead animals. Antigen is detected using two 'sandwich ELISAs' with polyclonal or monoclonal antibodies (Hamblin *et al.*, 1991; Laviada *et al.*, 1992). Both methods make use of the more conserved protein VP7 (segment 8) and

are noted for their high sensitive and specificity, with the added advantage that the results are available in 2-4 hours (Rubio *et al.*, 1998). Chicken egg yolk antibodies (IgY) have also been successfully used in a double-antibody sandwich setup to detect all nine serotypes (du Plessis *et al.*, 1999). The protocol is available from the OIE Manual (OIE, 2004a) and uses a biotin-avidin/peroxidase system for detection.

Virus Neutralisation (VN)

Serotyping the virus has important epidemiological functions. Virus Neutralisation (VN) is the traditional test (Howell, 1962; Mellor & Hamblin, 2004). However, this very tedious and time-consuming procedure will take at least five days for a result, often up to two weeks, in order to grow up the respective cells. VN requires live, replicating virions and uses mammalian cells or suckling mice (Hazrati & Ozawa, 1965).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Other means of identifying the virus include the reverse transcription-polymerase chain reaction (RT-PCR). Infectious disease diagnosis has been revolutionised by PCR-based molecular detection systems (Eaton & White, 2004). Various PCR assays have been developed since the mid-1990's for AHSV. The first RT-PCR assay was developed in 1993 and used primers to amplify segment 5 (NS1) from an attenuated strain in Vero cells (Mizukoshi *et al.*, 1994). Other protocols used group-specific primers for either VP3 (Sakamoto *et al.*, 1994) or VP7 (Zientara *et al.*, 1995b). The OIE recommends the protocols developed by Stone-Marschat *et al.* (1994), Zientara *et al.* (1994) and Laviada *et al.* (1997) which amplify the NS2 gene. As AHSV is a dsRNA virus, reverse transcriptase-PCR (RT-PCR) must be performed where the RNA is reverse transcribed to complementary DNA (cDNA) first, followed by the amplification process. RNA is isolated using either the phenol : chloroform method or a commercial kit can be used as successfully. The OIE Manual contains the particulars for the PCR reaction. Zientara *et al.* (1995a) was able to partially differentiate the nine serotypes using restriction patterns following PCR amplification of Segment 10. In 2000, the first RT-PCR to distinguish all nine serotypes was developed, amplifying sequences from Segment 2 (VP2), the most serotype specific segment. According to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, the results showed a perfect correlation with a virus neutralisation test. However, nine pairs of primers were used in nine separate reactions (Sailleau *et al.*, 2000; OIE, 2004a), making the assay costly

and time-consuming. Molecular probes have also been used in conjunction with RT-PCR (Moulay *et al.*, 1995).

PCR has additional advantages in that it can detect the virus during the early stages of viraemia and in samples that have been poorly preserved, unlike viral isolation techniques (Sailleau *et al.*, 1997). Some caveats exist when performing PCR on blood samples. It has been reported that PCR may be inhibited by components of haem, and anti-coagulants such as heparin and EDTA although in studies of PCR on other organisms this was not found to be the case (Holodniy *et al.*, 1991; Barker *et al.*, 1992). Washing the blood samples at least twice appears to eliminate the inhibitory effects previously described (Sailleau *et al.*, 1997). Sailleau *et al.* (1997) further reports that PCR achieves results in less than 24 hours as opposed to the more traditional viral isolation methods where it may take up to 15 days for a positive result in less virulent strains.

PCR is now routinely used in most diagnostic laboratories. In these settings, sample volume and cost become a priority. Multiplex PCR provides the answer by allowing more than one target sequence to be amplified simultaneously. However, the optimisation of multiplex PCRs can be complicated. In terms of diagnostic virology and differential diagnosis, multiplex PCR has many obvious advantages as a rapid and convenient screening assay, especially when considering multiple products (Rachlin *et al.;* Henegariu *et al.*, 1997; Elnifro *et al.*, 2000).

2.8.3 Differential Diagnosis

It is important to distinguish AHS from other similarly presenting diseases as their consequences differ greatly. AHS shares many of the clinical signs and symptoms that are seen in the closely related *Orbivirus*, equine encephalosis virus (EEV). The diseases have similar epidemiological patterns and occur simultaneously in South Africa, both being vectored by *Culicoides*, although mortality is higher for AHS (Coetzer & Erasmus, 1994; Lord *et al.*, 2002; Venter *et al.*, 2002; Mellor & Hamblin, 2004). AHSV and EEV may also occur simultaneously in the same animal (Mellor & Hamblin, 2004; Howell, *Personal Communication*). A standardised test must be used to confirm either diagnosis. An indirect sandwich ELISA has been developed to indentify EEV antigen (Crafford *et al.*, 2003) and should be used in conjunction with tests to

determine the presence of AHS virus to determine whether a co-infection is present. In addition, the transmission dynamics of these two closely related viruses are not fully understood and additional data on their outbreak will assist in comprehending the geographical variation in transmission (Lord *et al.*, 2002)

Other differential diagnoses for AHS include babesiosis, purpura haemorrhagica, equine viral arteritis, equine infectious anaemia and equine morbillivirus pneumonia (OIE, 2004a). Purpura haemorrhagica and equine viral arteritis share similar symptoms to the pulmonary form of AHS (Coetzer & Erasmus, 1994).

2.9 Proposed Research Methodology

2.9.1 Research Methods

An accurate and rapid diagnosis of equine infectious diseases has always been a priority for researchers and veterinarians alike throughout the ages. An early detection of the causative agent has immediate benefits that includes applying the correct treatment regime for the animal, notifying authorities in the case of notifiable diseases, implementing suitable control measures to prevent further spread of the disease and possibly using advanced targeted treatment strategies. The last two decades have seen a shift in the pursuit for rapid diagnostics from classical microbiology to molecular biology based techniques, such as nucleic acid detection, particularly the polymerase chain reaction (PCR). The applicability of PCR has increased dramatically by the advent of real-time or quantitative PCR (Desmettre, 1999; Powell, 2000; Wolk *et al.*, 2001; Pusterla *et al.*, 2006).

2.9.2 Propagating the AHS virus

Various methods have evolved that aim to multiply viruses to sufficient quantities so that they may be studied. Most viruses can now be propagated using artificial cell culture methods. Another method that was popular before the advent of cell culture was propagation in embryonated chicken eggs (ECE) that provided a sterile vessel for a virus to multiply. Both ECE and cell culture also provide the means to perform viral isolations for various diagnostic assays (Eaton & White, 2004). Newcastle disease virus of chickens is still isolated using ECE (Bouzari & Spardbrow, 2006; Maharaj, *Personal Communication*). The bluetongue virus is commonly propagated using ECE

via the intravascular route and is reported to be more sensitive than cell culture (Hosseini *et al.*, 1998; Clavijo *et al.*, 2000). The equine influenza virus and the swine influenza virus (single-stranded RNA virus) have also been found to be more suited to propagation in ECE rather than cell culture (Clavijo *et al.*, 2002; Quinlivan *et al.*, 2004). In addition, it has been reported that chicken egg yolk has stabilising properties for reverse transcriptase reactions and may protect viral particles from freeze-thaw cycles (Gazit *et al.*, 1978).

Initially, the AHS virus was successfully propagated in the brains of mice. However, this posed some problems in that large numbers of mice needed to be maintained, many of the mice would die and it was a labour intensive process. Alternative methods were therefore sought, embryonated chicken eggs being one of the early alternatives. Goldsmit (1967) successfully inoculated embryonated chicken eggs via the yolk sac route and recorded the distribution of the virus between extra-embryonic fluids and the embryo itself. The head contained the highest titre of both the neurotropic (Serotypes 1, 2, 3, 4, 5 and 6) and viscerotropic strains (Serotypes 7, 8, 9), while the viscerotropic strain was more widely distributed throughout the embryo and extra-embryonic fluids (Howell, 1962; Goldsmit, 1967).

Cell culture methods using monkey kidney cells (such as Vero cells) were developed in the 1960's (Ozawa *et al.*, 1965; Ozawa & Bahrami, 1966; Hazrati & Ozawa, 1968; Mirchamsy & Taslimi, 1968; Breese & Ozawa, 1969) and are currently the most popular.

2.9.3 Overview of the Polymerase Chain Reaction (PCR)

PCR is an *in vitro* cyclical method of rapidly amplifying nucleic acids. The basic components of PCR are two primers, a mixture of all four deoxynucleotides (dATP, dTTP, dGTP and dCTP), a heat-stable polymerase and a suitable buffer. In the simplest of terms, double stranded DNA is denatured, the primers anneal specifically to the DNA, a polymerase lengthens the sequence and the cycle begins again. All the steps are temperature dependent and each PCR must be optimised for this. Usually, the amplified nucleic acids are resolved in agarose gel and stained using ethidium bromide (Pusterla *et al.*, 2006) (Figure 2.12).

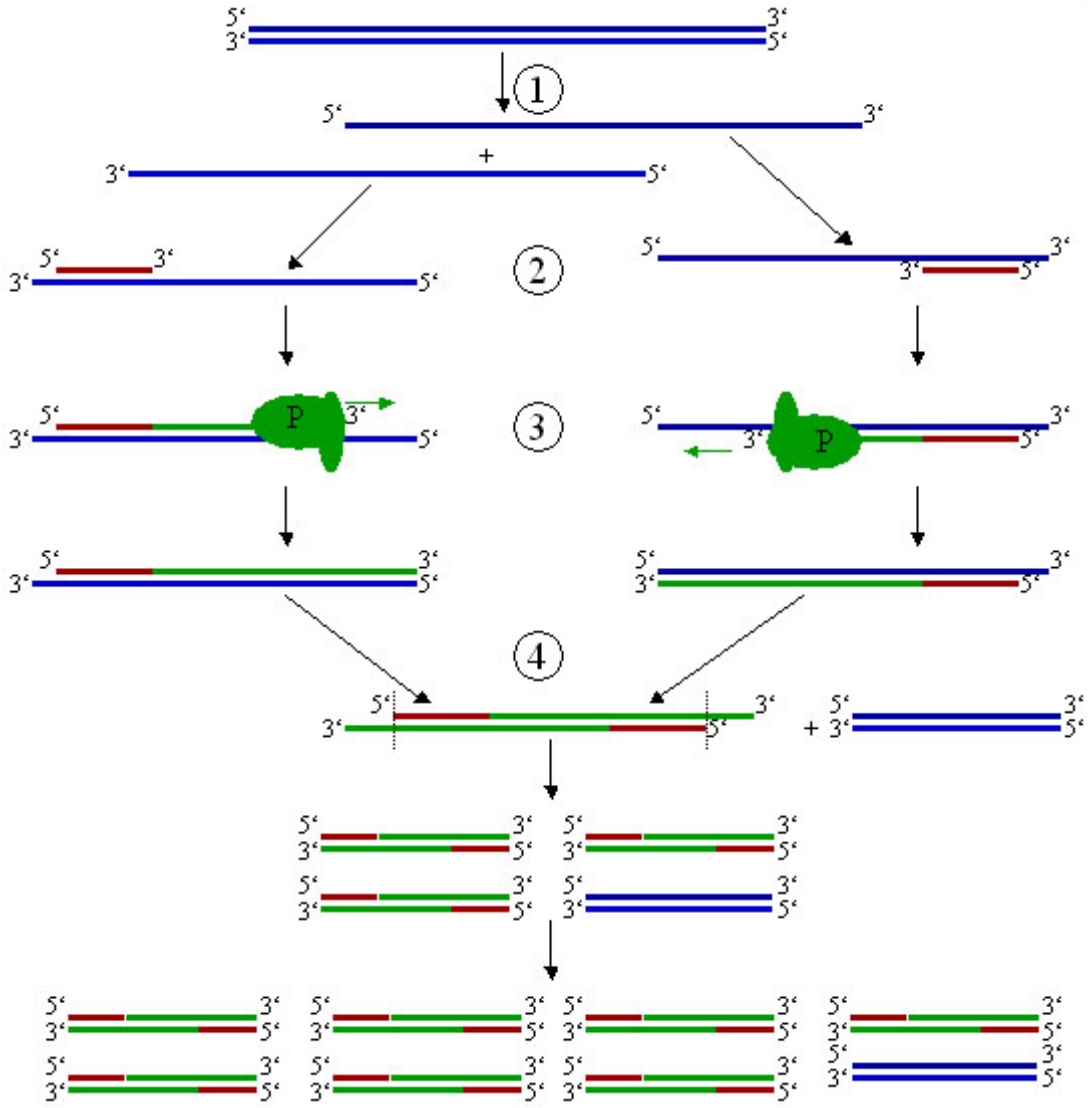

Figure 2.12: Simplified overview of the polymerase chain reaction.
(1) The parental strands are denatured at 94-96°C. (2) Primers anneal to the single strands at a calculated, specific temperature. (3) The DNA polymerase elongates the sequences at 72°C. (4) The resulting DNA strands form the template for the next round of amplification. (Rice, 2009).

2.9.4 Advantages and Disadvantages of PCR

Advantages

- Identification of slow-growing, difficult-to-cultivate or non-cultivatable organisms (Pusterla *et al.*, 2006).
- Clinical situations where conventional microbiology is inadequate, time-consuming or labour-intensive (e.g. cell culture), difficult or hazardous (Pusterla *et al.*, 2006).

- The rapid output of PCR results provides a means of limiting the spread of highly contagious pathogens (Pusterla *et al.*, 2006).
- Results available in less than 24 hours (Stone-Marschat *et al.*, 1994)

Disadvantages

- Viewing PCR products using gel electrophoresis in conventional protocols carries an inherent risk of product carryover from the 'master mix' leading to false positives (Pusterla *et al.*, 2006).
- The high sensitivity of PCR involves the risk of false positives (Stone-Marschat *et al.*, 1994)

Alternatives to PCR for nucleic acid amplification exist, and include, among others, loop-mediated isothermal amplification (LAMP) (Notomi *et al.*, 2000; Rodriguez-Sanchez *et al.*, 2008b). This novel method has as its primary advantage, static temperature ranges for its amplification process whereby various chains of stem loop structures are produced. As a result, only basic laboratory equipment is required.

2.9.5 Reverse Transcription PCR (RT-PCR)

RNA cannot be used as a template under standard PCR conditions. It must first undergo a transcription process to convert the RNA stands into complementary DNA (cDNA). This is usually achieved by a preliminary step involving a reverse transcriptase. From this point on, PCR continues as normal. In single tube, one-step RT-PCR protocols, the synthesis of cDNA and PCR occur sequentially but uninterrupted (Lee *et al.*, 1994). In most RT-PCR reactions, a reverse transcriptase is used to transcribe RNA into DNA and then a DNA polymerase synthesises the DNA amplicons. However, should an enzyme be available that will perform both functions, the entire procedure becomes many times simpler. The *Tth* DNA polymerase is an enzyme that has been successfully used to detect small quantities of mRNA. *Tth* DNA polymerase has an inherent reverse transcriptase activity and has also been reported to be able to work directly without time-consuming RNA isolation steps either (Chiocchia & Smith, 1997). In addition, *Tth* DNA polymerase was shown to work in the presence of common PCR inhibitors (Löfström *et al.*, 2004).

2.9.6 Using RT-PCR to detect AHS

A number of researchers have developed PCR assays to detect the AHS virus since the early 1990's and a brief overview of their experimental designs and differences are described below.

2.9.6.1 Isolation of AHSV genomic material

The double stranded RNA genome of the AHS virus that is the target of a PCR would ordinarily need to be isolated from the virus structure and surrounding biological components. In some cases it may be possible to perform a PCR on crude samples which would drastically reduce the cost of the test (Watson, *Personal Communication*). Stone-Marschat *et al.* (1994) purified viral dsRNA from infected Vero cell lysates by phenol extraction and lithium chloride precipitation based on the method of Clarke and McCrae (1981). Zientara *et al*. (1995b) isolated total RNA from cell cultures and spleen tissue samples using the guanidinium-thiocyanate-phenol-chloroform method of Chomczynski & Sacchi (1987). Commercial kits based on this method are now available such as the TRIzol™ group of reagents (Rodriguez-Sanchez *et al.*, 2008a). The quantity of AHSV genomic dsRNA can be determined spectrophotometrically at 260 nm (Wade-Evans *et al.*, 1990).

In terms of field samples, it has been reported that the AHS viral genome was successfully extracted and purified from clotted blood. This has the potential to reduce costs further since special blood collection vials containing anti-coagulants may no longer be needed (Fasina, 2008; Fasina *et al.*, 2008). In addition, direct PCR from whole blood may be possible by modifying the initial denaturation steps (Mercier *et al.*, 1990; McCusker *et al.*, 1992).

However, the possibility exists to eliminate viral RNA extraction procedures by using specially prepared filter paper (FTA® cards). In 1997, this was achieved with the Human Immunodeficiency Virus (HIV) Type 1. In addition, RNA levels on the filter paper had not decreased after two weeks at 20°C and three days at 37°C. This has important consequences for field studies and the development of field applicable diagnostic assays (Cassol *et al.*, 1997). FTA cards have also been examined and for their ability to inactivate pathogens and for their storage ability and stability of nucleic acids, all of which revealed promising results for reducing field assay costs (Roy &

Nassuth, 2005; Purvis *et al.*, 2006). In 2007, real-time RT-PCR was performed on RNA porcine reproductive and respiratory virus using FTA® cards (Inoue *et al.*, 2007). By using a higher pH PCR buffer, Bu *et al.* (2008) were able to amplify genomic DNA directly from blood that had dried on the filter paper. This has an enormous potential to reduce costs involved in such diagnostic assays, as expensive reagents to extract genomic material will no longer be needed.

The extraction of viral RNA from the OBP manufactured freeze-dried vaccines may also be necessary. This has been achieved previously, albeit with an avian dsRNA reovirus (Bruhn *et al.*, 2005)

2.9.6.2 Selection of primers

Short, complementary DNA sequences, or oligonucleotides, designed to anneal to target DNA are termed primers. Primers are selected to amplify a specific sequence. There is a variety of software programs available that choose the most appropriate primers for a sequence such as the free, internet-based Primer3 (Rozen & Skaletsky, 2000) and *Primaclade* (Gadberry *et al.*, 2005). There are a few general characteristics of primers that should be adhered to (Rybicki, 2001):

- The primers should be between 17-28 nucleotide bases in length
- The composition of the guanidine and cytosine (GC) bases should be 50-60%
- The 3′ end of the primer should end in a guanidine or cytosine. Guanidine and cytosine are joined by three hydrogen bonds and are therefore stronger than the double hydrogen bonds of an adenine-thymine pairing.
- The melting temperatures should be between 55-80°C
- A series of three or more Gs or Cs at the 3′-ends of primers should be avoided. As they are more stable, they may mis-prime at G or C-rich sequences
- Complementary 3′-ends of primer pairs should be avoided. This may result in primer dimers forming
- Primers that contain self-complementary regions will form secondary structure and be prevented from annealing

Previous workers have selected a range of sequences from different genome segments of the AHSV. The NS2 gene was used in the first PCR published to detect the AHS virus (1994). The NS2 gene has high sequence similarity within the

serogroup, but was divergent enough among serogroups not to detect other *Orbiviruses.* A single-tube RT-PCR was developed targeting regions on the VP7 and NS3 gene a year later and all nine serotypes were detected (Zientara *et al.*, 1995b). Following on from that work, the NS3 gene (Segment 10) was used to differentiate the nine serotypes using restriction fragment length polymorphism (RFLP). This novel method used restriction enzymes that hydrolysed the amplified regions resulting in specific, unique patterns on agarose gels (Zientara *et al.*, 1995a). In 1997, VP7 was targeted again, coupled with a dot-blot hybridisation technique (Sailleau *et al.*, 1997). In 2000, all nine serotypes were individually identified using 15 different primers in different combinations, but in nine separately optimised PCR runs (Sailleau *et al.*, 2000). In South Africa, the first PCR assay was developed in 2004. It serotyped the virus using 16 primers under identical reaction conditions (Koekemoer & van Dijk, 2004). However, it required lengthy post-PCR analysis. Rodriguez-Sanchez (2008a) used the NS1 gene as a target and combined it with gel-based techniques. Most recently, the VP7 gene was used to develop an assay for AHS coupled with probe-based technologies (Fernández-Pinero *et al.*, 2009).

2.9.6.3 cDNA synthesis

PCR can only work from DNA templates, as the polymerase used is a DNA polymerase. RNA therefore needs to be transcribed into DNA (complementary DNA or cDNA). When double stranded RNA is the initial nucleic material, such as is the case for the AHS virus, it must be denatured so that cDNA can be synthesised from it. This is achieved by either heat denaturation or adding a methyl mercuric hydroxide solution to dsRNA material (Wade-Evans *et al.*, 1990; Zientara *et al.*, 1995b) in the presence of each primer and incubating at room temperature for 10 minutes. Compared to heat denaturation, methyl mercuric hydroxide increases the sensitivity of RT-PCR by ten-fold (Wilson & Chase, 1993), although heat denaturation is the most often used in recent years. The now single stranded RNA can then be combined with a solution of each deoxynucleotide triphosphate (dNTP) (i.e. dATP, dTTP, dGTP and dCTP) in a suitable buffer and a reverse transcriptase. The solution will then undergo a series of temperature changes for example: incubation at 37°C for 1 hour, heated to 95°C for 5 minutes to denature the reverse transcriptase and chilled on ice for 5 minutes. The cDNA is stored at -20°C (Stone-Marschat *et al.*, 1994).

2.9.6.4 PCR

The cDNA template can now be subjected to standard PCR protocols. One of the OIE recommended protocols is as follows: 30 cycles of 95°C for 1 minute, 42° for 1 minute, 70°C for 2 minutes and followed by 70°C for 10 minutes (Stone-Marschat *et al.*, 1994). Articles published recently have tended towards kit-based PCR protocols such as the Brilliant® QRT-PCR Master Mix One-Step kit (Rodriguez-Sanchez *et al.*, 2008a), the Applied Biosystems GeneAmp Gold RNA PCR core kit (Quan *et al.*, 2008) or the Qiagen One Step RT-PCR kit (Fernández-Pinero *et al.*, 2009).

2.9.6.4.1 Real-time fluorogenic RT-PCR

To overcome the shortfalls of standard PCR, such as the gel-based nature of results and the increased time that this takes, Agüero *et al.* (2008) developed the first real-time fluorogenic RT-PCR for AHSV. This method was based on a TaqMan® probe and was directed towards Segment 7 of the AHSV genome. The authors claimed a 1000-fold increase in sensitivity compared to the OIE referenced method. TaqMan® probes are, unfortunately, not a cost-effective option. Hybridisation probes have also been used to serotype AHS isolates, and are an improvement on virus neutralisation assays (Koekemoer *et al.*, 2000).

2.9.6.4.2 Nested RT-PCR

A nested RT-PCR has been developed with equal sensitivity to the real-time fluorogenic RT-PCR described above (Aradaib, 2009). Remarkably, the authors claim that the RT-PCR that they describe can detect as little as 0.1 fg of viral RNA, equivalent to six viral particles. In terms of a rapid, cost-effective assay, this assay was also successfully used directly on clinical samples (blood, lungs, liver, and spleen). However, this method involves two PCR reactions that offset the time saved by the absence of RNA extraction procedures.

2.9.7 Using RT-PCR to detect other Orbiviruses

RT-PCR has been used successfully to amplify genomic material from other Orbiviruses. These include the bluetongue virus (Aradaib *et al.*, 1998; Johnson *et al.*, 2000; Billinis *et al.*, 2001; Abdalla *et al.*, 2003; Aradaib *et al.*, 2003a; Aradaib *et al.*, 2003b; Anthony *et al.*, 2004; Ohashi *et al.*, 2004; Orru *et al.*, 2004; Zientara *et al.*,

2004; Jimenez-Clavero *et al.*, 2006; Monaco *et al.*, 2006); epizootic haemorrhagic disease virus (Abdalla *et al.*, 2002; Aradaib *et al.*, 2003a; Aradaib *et al.*, 2003b) and the Chuzan and Ibaraki viruses (Ohashi *et al.*, 2004). Importantly the sensitivity of these RT-PCR assays compares very favourably to the conventionally accepted norm of virus isolation (Abdalla *et al.*, 2002).

In a study done in 1995 on epizootic haemorrhagic disease virus (Orbivirus group), PCR assays were already being identified as equal to, if not superior to the "cumbersome and time-consuming" virus neutralisation assays (Aradaib *et al.*, 1995).

2.9.8 High Resolution Melt (HRM) Analysis

High resolution melting was introduced in 2002 through the collaborative efforts of academia (University of Utah) and industry (Idaho Technology) to detect genetic variations of double stranded DNA (Reed *et al.*, 2007). Various methods have previously been developed to detect DNA sequence variation of PCR products. However, these involve extra, lengthy processing and separation steps subsequent to the PCR run and include additional apparatus (Reed *et al.*, 2007). Gundry *et al*. (2003) described the ability of melting temperatures to distinguish unique variants in a homogenous, closed tube procedure performed automatically after PCR. The denaturation of double stranded DNA into two separate strands is a fundamental property of DNA when subjected to heat (Erali *et al.*, 2008). HRM requires normal PCR reagents, a fluorescing dsDNA dye and approximately 10-15 minutes of closed-tube, post PCR analysis (Reed & Wittwer, 2004). Reed *et al.* (2007) considers HRM the simplest method of determining sequence variation and recognises its increasing popularity.

Historically, DNA melting was monitored by UV absorbance, requiring microgram amounts and very slow melting rates. HRM, on the other hand, requires only nanogram amounts, provided by PCR products (Reed *et al.*, 2007). Earlier HRM applications used primers as well as various probes. However, these proved to be too limiting for routine use (Wittwer *et al.*, 2003; Liew *et al.*, 2004). Following standard PCR, which results in a high copy number of a purified amplicon, HRM analysis is an advancement of previous melting analyses based on DNA denaturation or dissociation. It is based on the release of a DNA-intercalating fluorescent dye that is released from dsDNA as it is denatured

or dissociated into ssDNA with increasing temperature (Figure 2.13). The melt curve is generated by heating the sample through a range of temperatures as fluorescence data is continuously collected. At low temperatures, the dsDNA fluoresces strongly. As the temperature is increased, so the dye is released from the dsDNA structures, and at a characteristic point, the fluorescence drops rapidly, indicating the dissociation of the dsDNA into single strands (the melting temperature of the DNA (T_m)).

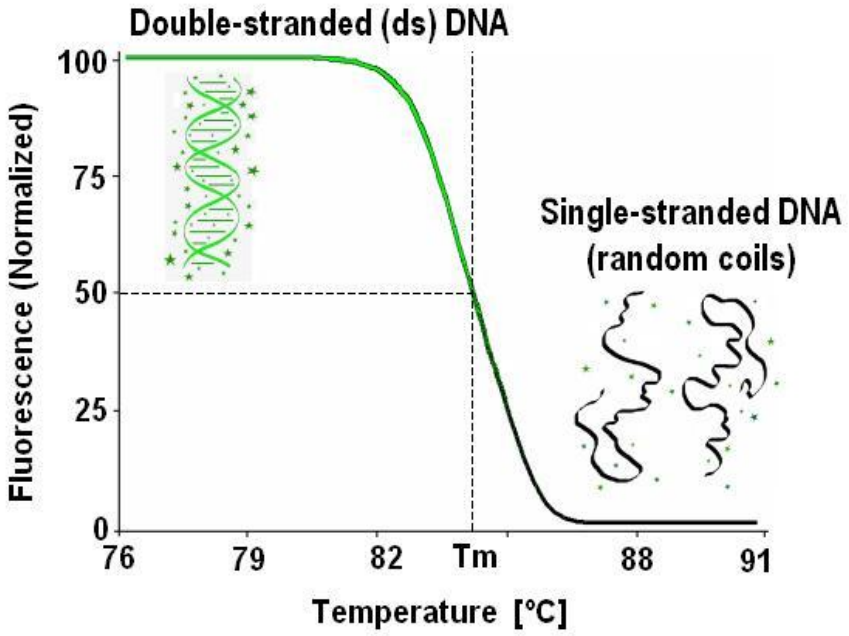

Figure 2.13: Simplified melt profile demonstrating the release of the fluorescent dye as the DNA moves from a double-stranded structure to a single-stranded one. The dye is intercalated into the strands of the dsDNA at low temperaures, where it fluoresces strongly. As the temperature is increased, so the dsDNA structure begins to dissociate into ssDNA and the dye is released and no longer fluoresces.

The characteristic melting of a DNA sequence is defined by the relative stabilities and kinetic melting rates that are dependent on sequence length, GC content and sequence complementarity, which are unique among genetic variants of the same genome segment. Based on this, HRM has a number of applications including mutation detection, genotyping and species identification (Gundry *et al.*, 2003; Corbett, 2006; Hubbart *et al.*, 2007; Reed *et al.*, 2007). The decrease in fluorescence is measured with a high degree of optical and thermal precision and is analysed *in silico* (Corbett, 2006). HRM is simple, cost-effective and requires no post-PCR processing such as agarose gel electrophoresis. It also compares favourably with other similar, expensive techniques (Corbett, 2006; White & Potts, 2006).

Part of the recent success of HRM is the introduction of 3rd generation fluorescent dsDNA dyes. SYTO®9 (Invitrogen Corp., Carlsbad, CA), LCGreen® (Idaho Technologies, Salt Lake City, UT) and EvaGreen® (Biotium Inc, Hayward, CA) have lower toxicity levels than previous, older dyes and, as such, can be used at higher concentrations that ensure saturation of the dsDNA. Previous dyes had to be used at a lower concentration resulting in low levels of saturation and low sensitivity (Gundry *et al.*, 2003; Wittwer *et al.*, 2003; Corbett, 2006). Previous dyes also had a tendency to preferentially bind to sequences with a higher melting temperature (T_m) and GC-rich regions (Zhou *et al.*, 2004). Older dyes, such as SYBR Green I, had to be optimised further when used in standard PCR buffers by the addition of dimethyl sulfoxide (DMSO), bovine serum albumin or Triton X-100. In addition, the dye was reported to inhibit the PCR reaction without an increase in $MgCl_2$ and interferes in multiplex PCRs. SYTO 9 supports PCR in a wide range of applications, produces robust melting curves unaffected by DNA or dye concentration and can be used in multiplex PCR reactions (Monis *et al.*, 2005).

The sensitivity of HRM is evident in its ability to detect the smallest genetic change such as single base changes (single nucleotide polymorphisms, SNPs). In general, the greater the number of changes, the easier they are to detect using HRM (Corbett, 2006; White & Potts, 2006).

HRM is best performed on highly pure PCR products of less than 250 base pairs (bp), although 44-304 bp amplicons have been analysed previously (Gundry *et al.*, 2003; Corbett, 2006). Amplicons of up to 1000 bp have also been successfully analysed using HRM (Reed & Wittwer, 2004) The largest recorded amplicon for HRM application has been 1330 bp (Chateigner-Boutin & Small, 2007). The larger the amplicon, the lower the resolution as the difference between the sequences decreases. This can be overcome to some extent by melting at slower rates at the expense of an extended analysis time (Gundry *et al.*, 2003). Additionally, certain sequence motifs, secondary structures, localised regions of high or low GC content or repeat sequences can all affect the results unpredictably (Corbett, 2006).

An HRM analysis must be preceded by a good quality and accurate PCR, as would be a normal real-time PCR assay. This includes the design phase where the target sequence and primers are identified and the reactions are set up. Interestingly, HRM

has been successfully performed on samples from dried blood spots (Gundry *et al.*, 2003; Corbett, 2006).

The post-PCR/HRM analysis results are viewed as fluorescence versus temperature graphs and mathematical derivatives of this (Figure 2.14). The graphs are normalised for each sample by defining linear baselines before and after the melting transition (Figure 2.14B). The fluorescence for each acquisition within the sample is calculated as a percentage between the top and bottom baselines at the acquisition temperature (Figure 2.14C) (Gundry *et al.*, 2003). Normalised curves represent the basic interpretation for sequence variation and are based on curve shifting, shape change and position (Wittwer *et al.*, 2003). Wittwer *et al.* (2003) also described fluorescence difference as a useful method of differentiation (Figure 2.14D). This allows better visual grouping of genotypes. One melting curve is chosen as the reference and the rest are plotted against it as a difference. The HRM software now also allows for the automatic calling of genotypes with a set confidence interval (Corbett, 2006).

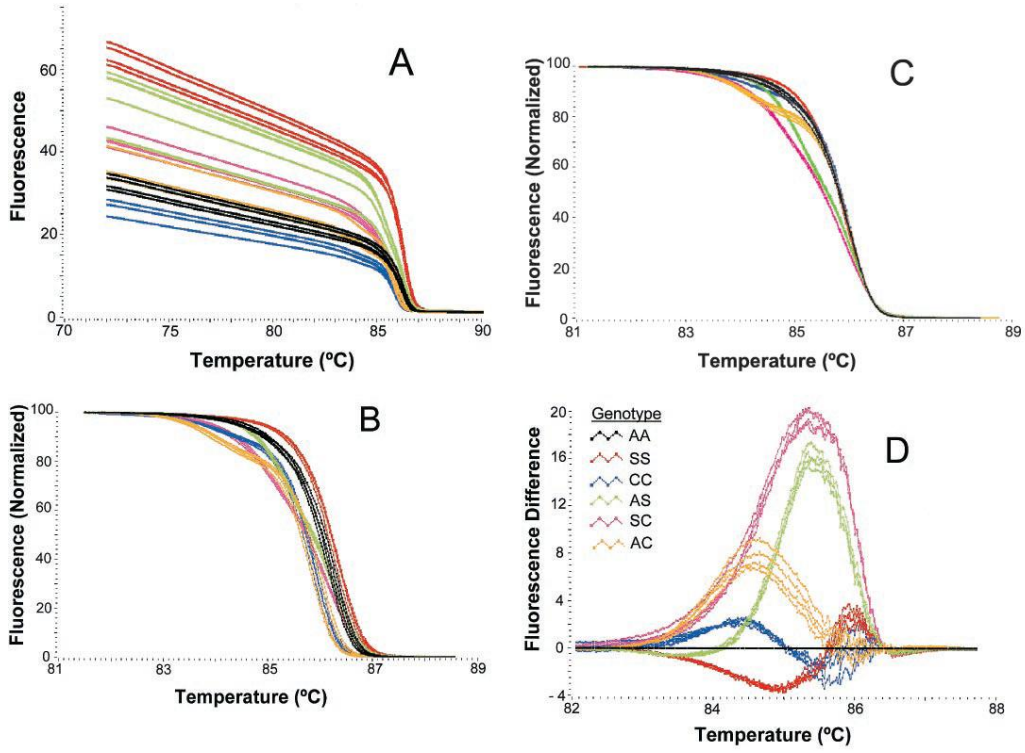

Figure 2.14: Variations of the interpretations of the fluorescent data post HRM.
A) Raw fluorescent data in real-time. B) Normalised fluorescence. C) Temperature shifted normalised fluorescence. D) Difference plots of the normalised, temperature shifted data (Wittwer *et al.*, 2003).

In studies that used HRM to detect mutations, DNA sequencing confirmed that normal melting curves correlated with normal DNA sequences and abnormal melting curves correlated with abnormal DNA sequences (Willmore *et al.*, 2004). In a study performed in 2004, large sequence differences were found to be detectable by HRM analysis (Vaughn & Elenitoba-Johnson, 2004). Previously, only single point mutations had been studied. Using HRM analysis, amplicons of 335 to 431 bp in length were analysed for insertions of 6-102 bp in length. 100% concordance was achieved compared to more standard methods such as capillary electrophoresis-based fragment analysis, temperature gradient capillary electrophoresis detection and sequencing. HRM analysis was also found to be effective at detecting mutations across the length of the amplicon, despite the mutations occurring at various positions.

Developing technically simple and reliable methods for detecting sequence variations in related genes has become an important goal of molecular diagnostics. Although DNA sequencing still remains the “gold standard”, it is significantly labour- and time-intensive for clinical, routine use (Highsmith, 2004).

Various programs have been developed that have attempted to produce melt curves entirely *in silico*, such as POLAND and MELTSIM. Although the programs were successful in accurately typing the samples, it was suggested that they were best suited to assay design (Rasmussen *et al.*, 2007).

2.9.9 Species differentiation using HRMA

A review of the literature pertaining to HRM applications will reveal that most HRM applications have sought the detection of single point mutations. Genotyping was achieved in platelet antigens using unlabelled probes and HRM (Liew *et al.*, 2006). However, in 2006, Robinson *et al.* (2006) successfully and reproducibly differentiated between species of the amoebaflagellate genus *Naegleria* using a single primer set. The sequences amplified were almost invariant in individual species, but divergent among species. The melting curves that resulted were distinguishable and unique for each species due to the differences seen in the positions and relative heights of the peaks (Figure 2.15). Feline caliciviruses (Helps *et al.*, 2002), *Cryptosporidium* species (Limor *et al.*, 2002; Tanriverdi *et al.*, 2002), *Leishmania* species (Nicolas *et al.*, 2002), Mycobacteria species (Odell *et al.*, 2005), *Plasmodium* species (Mangold *et al.*, 2005)

and *Camphylobacter jejuni* (Price *et al.*, 2007) have also all been successfully species-differentiated using HRMA.

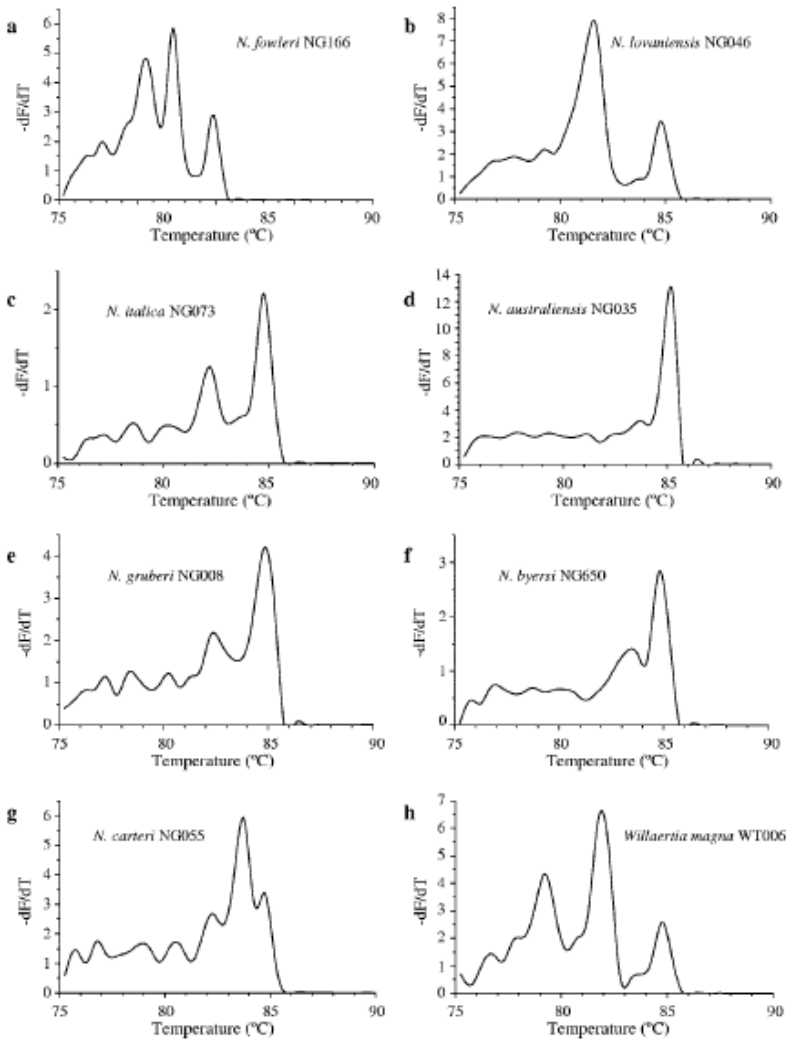

Figure 2.15: Melt curves obtained from *Naeglaria* species using HRM demonstrating unique positions and relative heights of peaks between the species. Each graph represents (Robinson *et al.*, 2006)

HRM analysis has been used to detect and identify 25 clinically important bacteria (Cheng *et al.*, 2006). As little as 1 pg of bacterial DNA was detectable. In addition, there appears to be little difference in the melting curves of DNA amplified from bacteria and bacterial suspensions. 25 bacterial species were studied, nine of them being identifiable from their characteristic melting curves. The remaining 16 species were grouped into four melting groups for further assessment. Twelve of these species were identified through a heteroduplex formation with a chosen reference species and produced unique melt curves. The remaining four undistinguishable species were

subjected to a second real-time PCR using a modified primer pair. All the species were able to be identified via their unique melting curves except two whose curves were undistinguishable.

More recently, HRM was used to distinguish *Bacillus anthracis* species using LC Green® I (Fortini *et al.*, 2007). In this study, while it was suggested that DNA concentration and quality were key factors in reproducible melting curves, it was found that by reducing the amount of DNA intercalating dye by half, the melting curves became more reproducible.

In 2009, the use of HRM combined with a unique mathematical model was used to differentiate strains of infectious bronchitis virus (IBV) on 230-436 bp products such that the most effective vaccination program could be applied (Figure 2.16). Rapid control of IBV outbreaks was mooted as a potential application of this assay (Hewson *et al.*, 2009).

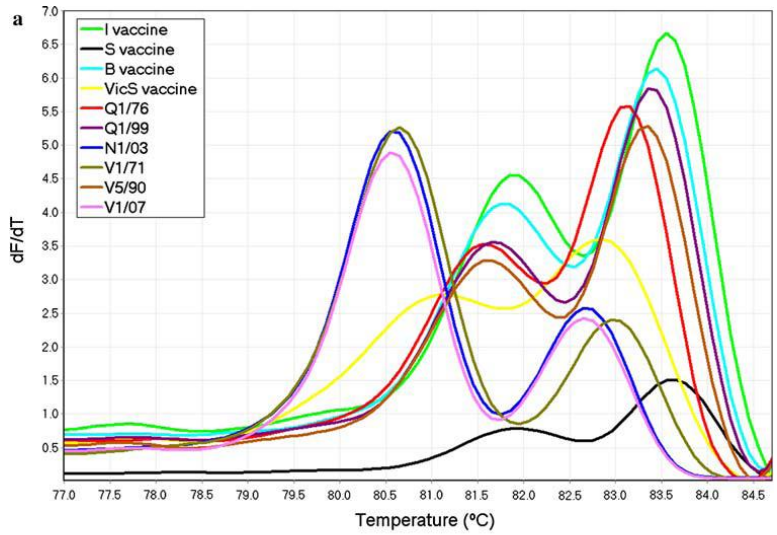

Figure 2.16: Conventional melt curves of various wild-type and vaccine strains of IBV (Hewson *et al.*, 2009)

Also in 2009, *Chlamydophila psittaci* species and *Bartonella* spp. were genotyped using HRM analysis (Mitchell *et al.*, 2009; Morick *et al.*, 2009).

In addition to species differentiation, characterisation of isolates has also been performed for the poultry pathogen *Mycoplasma synoviae.* Amplicons of approximately 400 bp were used to characterise 35 different strains into 10 profiles. Importantly, the

authors claimed that the HRM curve analysis is a 'rapid and effective technique that can be performed in a single test tube in less than two hours' (Jeffery *et al.*, 2007).

2.9.10 HRM Instrument Comparison

A study by Herrmann *et al.* (2006) compared the available instrumentation for DNA amplicon melting analysis. Nine instruments were compared, namely, the Prism® 7000 SDS and 7900HT (Applied Biosystems), iCycler iQ (Bio-Rad), SmartCycler® II (Cepheid), Rotor-Gene™ 3000 (Corbett Research), LightScanner® (Idaho Technology), HR-1™ (Idaho Technology) and the LightCycler 1.2 and LightCycler 2.0 (Roche). The Corbett Rotor-Gene™ 3000 (and its successor, the Rotor-Gene™ 6000) approaches HRM slightly differently to other machines. Melting is performed at a much slower rate such that the melting is performed step-wise, enabling more data points per second to be acquired and for the fluorescence to normalise before each reading. However, this does increase the noise ratio (Herrmann *et al.*, 2006; Reed *et al.*, 2007). It is also important to note that this study remains the only instrument comparison available.

2.9.11 HRM and probes

Melt curves can, however, be complicated to analyse and distinguish between closely related genotypes. The addition of unlabeled probes to the reaction mixture may provide additional information. The melt analysis will now include both the amplicon melting and the probe melting in two distinct phases. The probes are blocked at the 3' end and the PCR is run in an asymmetric fashion such that there is a limiting primer and an excess primer (Figure 2.17) (Graham *et al.*, 2005; Montgomery *et al.*, 2007; Erali *et al.*, 2008).

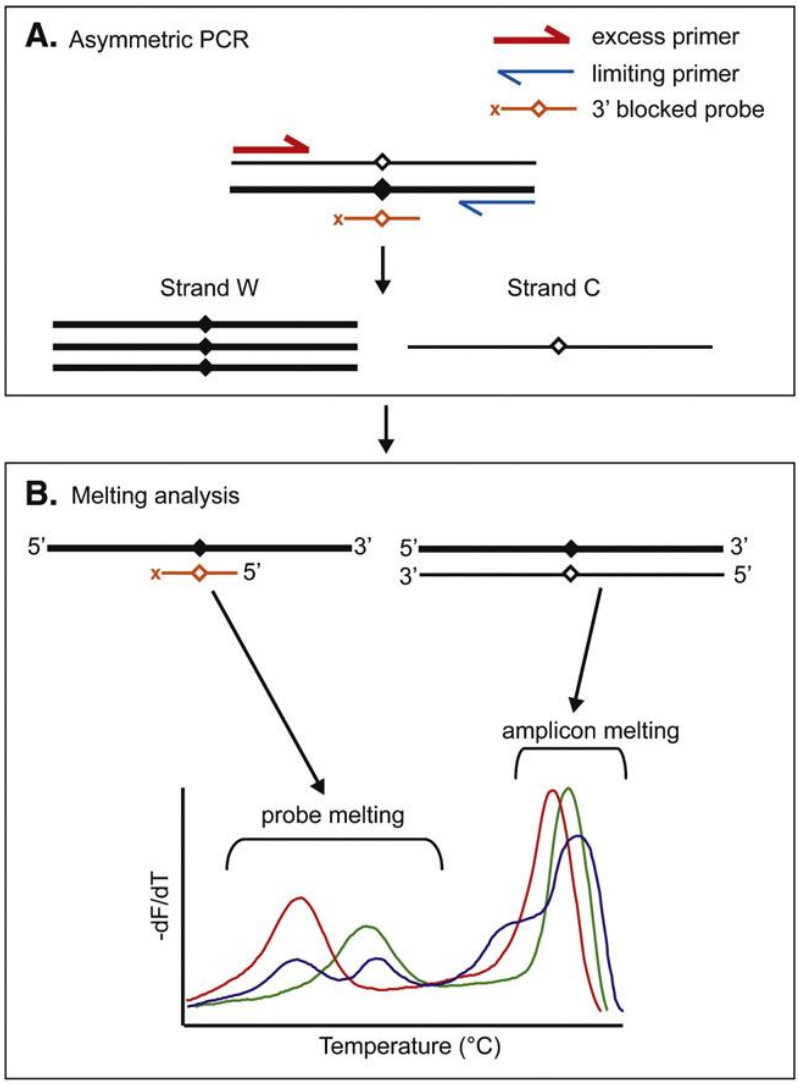

Figure 2.17: Asymmetric PCR with amplicon and unlabelled probe melting.
A) In asymmetric PCR, strand W is produced in excess while strand C is produced in a limited fashion. The excess strand W's are hybridised with the unlabelled probe and subsequently melted off. Strand C produces undergoes standard PCR reactions and produces full-length amplicons that are melted with the unlabelled probes. B) Both probe and amplicon melts are seen distinctly on the melt analysis (Erali *et al.*, 2008).

Real time RT-PCR and melt curve analysis were used to serotype the AHS virus in 2008 using Segment 2. However, it involved nine pairs of hybridisation probes and displayed some peak melting temperature shifts. Ultimately, it was found that this method was far from ideal for serotype detection and could not be regarded as a rapid and cost-effective assay. In addition the data did not appear to be statistically interrogated, nor was the possibility of targeting alternate regions discussed (Koekemoer, 2008).

2.10 Conclusion

African horse sickness has long plagued the equines inhabiting Southern Africa. The often-elite profile of sporting and racing horses tends to encourage the sidelining of rural subsistence and traction animals and those who rely on them. AHS may have socio-economic consequences for rural communities, but this has yet to be assessed. The high cost of research has led to a dearth of work being done in the field of AHS and the current knowledge about AHS is somewhat lacking in many respects. In order to counter this, rapid, cost-effective assays are crucial to enable a greater understanding of the disease, its epidemiology and its control. The assay that is proposed will provide a unique method for rapid serotyping of the African horse sickness virus.

The development of this assay, however, requires that certain preliminary investigations be carried out. These include the design of particular primer pairs, the propagation of the AHS virus, viral RNA extraction and the analysis of HRM curves following standard RT-PCR.

Chapter 3: PROPAGATION AND EXTRACTION OF AHS VIRAL RNA

3.1 Introduction

In terms of preliminary investigations for a rapid assay, known viral stocks are required from where copious amounts of RNA would be readily available. The extraction of viral RNA from inoculated ECE remains a common method, but is a laborious process with many extraneous circumstances. Cell culture represents the modern alternative in that it is quick and controlled. Propagating the AHS virus in monkey kidney cells, MS and Vero cultures have been reported as early as the 1960's (Ozawa *et al.*, 1965; Ozawa & Bahrami, 1966; Hazrati & Ozawa, 1968; Mirchamsy & Taslimi, 1968; Breese & Ozawa, 1969). The Vero cell line (Figure 3.1) originated from the kidney of an African green monkey in 1962 and is commonly indicated for the propagation of viruses (ATCC, 2009).

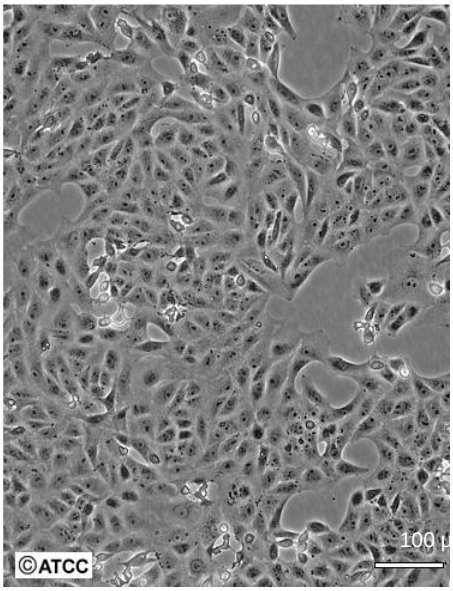

Figure 3.1: Micrograph of a high density monolayer of Vero cells cultured in EMEM containing 10% FBS (after ATCC, 2009).

The AHS virus was initially propagated successfully in the brains of mice. However, this posed some problems in that large numbers of mice needed to be maintained, many of the mice would die and it was a labour intensive process. Alternative methods were

therefore sought, embryonated chicken eggs being one of the early alternatives. Goldsmit (1967) successfully inoculated embryonated chicken eggs via the yolk sac route and recorded the distribution of the AHS virus between extra-embryonic fluids and the embryo itself. The head contained the highest titre of both the neurotropic (Serotypes 1, 2, 3, 4, 5 and 6) and viscerotropic strains (Serotypes 7, 8, 9), while the viscerotropic strain was more widely distributed throughout the embryo and extra-embryonic fluids. All serotypes, however, were distributed throughout the embryo and extra-embryonic fluids (Howell, 1962; Goldsmit, 1967). The extra-embryonic fluids (particularly the yolk sac) are the easiest to inoculate and harvest. The embryo will require specialised, trained personnel to inoculate and requires extra steps for harvesting (Taylor, 1952; Boorman *et al.*, 1975; Kelling & Schipper, 1976; Clavijo *et al.*, 2000)

Where laboratories are not equipped to deal with specialised cell culture techniques, embryonated chicken eggs (ECE's) provides a valuable alternative for the propagation of AHSV. ECE's provide a sterile vessel for a virus to multiply. Newcastle disease virus (Bouzari & Spardbrow, 2006; Maharaj, *Personal Communication*), bluetongue virus (Hosseini *et al.*, 1998; Clavijo *et al.*, 2000), equine influenza virus and swine influenza virus (Clavijo *et al.*, 2002; Quinlivan *et al.*, 2004) are still isolated using ECE. In addition, it has been reported that chicken egg yolk has stabilising properties for reverse transcriptase reactions and may protect viral particles from freeze-thaw cycles (Gazit *et al.*, 1978).

Additionally, in the absence of AHSV reference strains, virus can theoretically be obtained from the Onderstepoort Biological Products (OBP) manufactured AHS vaccine. Isolated virus preparations are required to produce PCR products, as can be easily obtained from inoculated ECE. This protocol was tested on vaccine live, attenuated virus produced by OBP to provide virus for RNA extraction and RT-PCR using TRIzol® (Invitrogen, Carlsbad, USA) as the industry standard. In order to study particular AHSV genome segments, original reference strains of the AHS virus were additionally propagated using Vero cells in a cell culture system.

3.2 Materials and Methods

3.2.1 Embryonated Chicken Egg Inoculation and Harvesting

Inoculation and harvesting work was carried out at the Allerton KwaZulu-Natal Provincial Veterinary Laboratory in Pietermaritzburg and was based on the work of Boorman *et al.* (1975). Ethics approval was obtained through the University of KwaZulu-Natal Animal Ethics committee. AHS vaccine vial 1 (Batch #181) produced by OBP and containing live attenuated strains of serotypes 1, 3 and 4 was used in the experimental inoculation of 11 specific pathogen free embryonated broiler chicken eggs, incubated under standard incubation procedures.

The vaccine contains at least 10^5 PFU per attenuated, freeze-dried pellet of AHS vial 1 (OIE, 2004). This was reconstituted with sterile, distilled water to produce dilutions of 1:2, 1:8, 1:64, such that the embryo survives to propagate the virus (Maharaj, *Personal Communication*). 200 µL of each dilution was inoculated into each egg. The eggs were set and incubated at 37°C, 60-65% humidity in an incubator, of which 11 were to be inoculated at seven days old. Harvesting of the ECE yolks occurred at 48 hours post-inoculation. The treatments are shown in Table 3.1.

Table 3.1: Treatment structure for the AHS virus inoculation of 7 day old embryonated broiler chicken eggs.

Treatment	Dilutions	# eggs
Control	no inoculation	1
Control	water only inoculation	1
Vaccine vial 1	1:2	3
	1:8	3
	1:64	3

To inoculate the eggs, they were placed air sac up in an egg tray and sterilised with a 0.01% (w/v) merthiolate solution. An eggshell punch was used to create a hole in the shell, without breaking the underlying membranes. Using a 21G, 1½" needle and a 1 mL syringe, 200 µL of inoculum was injected directly into the yolk sac. The hole was sealed with wood glue and the eggs returned to a lower temperature incubator and incubated at 35-36°C without turning.

Eggs were checked and candled daily for any deaths. Yolk sacs were harvested at 48 hours post-inoculation. Three eggs from each vaccine dilution treatment as well as one egg each from the control treatments were randomly selected. The eggs were placed air sac up on an egg tray and disinfected with a 0.01% (w/v) merthiolate solution (Maharaj, *Personal Communication*). The top of the egg was cracked open with forceps. Using the forceps, the embryo was immediately decapitated and removed along with the albumin such that only the yolk remained in the shell and it was extracted with a needle and syringe and placed in suitable storage vessels and frozen at -20°C. All the above procedures were carried out under BSL2 levels.

3.2.2 Vero Cell Culture

A 2 mL vial of Vero cells (passage #14) was split into 3 T75 flasks (75 cm^2) and 10 mL growth media added. Growth media contained: 10% foetal bovine serum (FBS) (Delta Bioproducts, Johannesburg, RSA); 1% Pen/Strep/Amphotericin B 100x (10,000 U penicillin/mL, 10,000 µg Streptomycin/mL, 25 µg Amphotericin B/mL) (Lonza, Walkersville, USA); 1% non-essential amino acid (NEAA) mixture 100x (Lonza, Walkersville, USA) and 1% L-glutamine (200 mM) (Lonza, Walkersville, USA) in Eagle's Minimum Essential Medium (EMEM) with EBSS and 25 mM Hepes and without L-glutamine.

At passage 20, nine T75 flasks with a confluent monolayer of Vero cells were selected for inoculation with the AHS virus reference strains provided in freeze-dried pellets (NICD). Details of the strains are contained in Table 3.2:

Table 3.2: Details of the AHS Virus Reference strains (NICD) for use in Vero cell culture for RNA viral isolation.

Serotype	Isolate	Year of original isolation	Year of last isolation
1	A501	1965	1997
2	OD	1965	1997
3	L	1965	1997
4	Vry 47/58	1965	1997
5	VH	1965	2000
6	114	1965	1997
7	Karen	1965	1997
8	18/60/22	1962	1998
9	AHS 9	1995	2000

The reference strains represent the original field virus isolates identified in the 1960's and are preserved as reference material in Reference Laboratories across the world. They also serve to confirm the genetic conservation of the virus through time. The reference strains at the NICD were received from Prof PG Howell (University of Pretoria) in 2000 who originally obtained the strains from the Onderstepoort Veterinary Institute and from the National Institute of Virology. The freeze-dried pellets obtained from the NICD are the product of a succession of mouse brain passages, baby hamster kidney (BHK) cell culture and Vero cell culture.

The freeze-dried pellets were resuspended in 250 µL of sterile phosphate buffered saline (PBS), aliquoted into 50 µL and stored at -70°C. To inoculate a T75 flask, 950 µL of growth media was added to a 50 µL aliquot (1:20 dilution) and transferred to a ± 90% confluent monolayer of Vero cells. A control flask was inoculated with 1 mL EMEM. The flasks were placed in a Gallenkamp Orbital Incubator (60 rpm) for 1 hour at 37°C, subsequently were overlayed with 10 mL growth media, and placed back into the incubator (40 rpm). Flasks were monitored daily for non-specific cytopathic effects (CPE) that involved the cells rounding and lifting off the surface of the flask (Paweska, *Personal Communication*). Flasks showed 90-100% CPE after 5-7 days post-inoculation and were harvested. The supernatant of approximately 10 mL was poured into a sterile 15 mL BD Falcon™ tube (BD Biosciences, San Jose, USA). The tubes were centrifuged at 2000 × *g* for 10 minutes at 4°C in a Beckman-Coulter Avanti® J-26XP centrifuge (JA-10 rotor). 10 mL of the resultant supernatant was removed and

transferred to a new tube and kept at 4°C. The pellet was resuspended in 2.5 mL of EMEM and frozen in liquid nitrogen followed by thawing at room temperature. The suspension was then subjected to a repeat centrifuge-freeze-thaw cycle. All of the supernatants were pooled consecutively and aliquoted into 1 mL volumes. They were subsequently stored at -70°C. All work involving the virus was performed according to BSL2 safety levels.

3.2.3 AHSV RNA Extraction

The extraction of viral RNA from the egg yolk and from the cell culture supernatants was achieved using TRIzol® LS Reagent (Invitrogen, Carlsbad, USA). Various variations to the standard protocol of Invitrogen (Carlsbad, USA) were used to extract viral RNA from the egg yolk as the standard protocol resulted in no RNA being extracted (Additional or amended steps for the yolk extraction are shown in italics).

3.2.3.1 Extraction from egg yolk

3.2.3.1.1 Homogenisation

250 µL of egg yolk sample and 750 µL of TRIzol® LS Reagent were combined in a microcentrifuge tube to achieve a minimum ratio of one part sample to three parts TRIzol® LS Reagent. The sample was homogenised by passing several times through a Gilson Pipetman® P1000 pipette. *The initial yolk sample was diluted 1:1 with DEPC-water and compared to non-diluted samples (Invitrogen, 2007; Watson, Personal Communication). Homogenisation was also achieved using a 21G needle (Watson, Personal Communication).*

An extra centrifugation step was added after homogenisation to remove any insoluble material from the yolk. The homogenised sample was centrifuged at 12,000 × g *for 10 minutes at 4°C. Three phases resulted and all but the top, fatty layer were removed for downstream steps (Invitrogen, 2007).*

3.2.3.1.2 Phase Separation

The homogenised samples were incubated for 5 minutes at room temperature (15 to 30°C). 150 µL of chloroform was added at a ratio of 0.2 mL of chloroform per 1 mL of TRIzol® LS Reagent. The tubes were capped securely and vigorously shaken for 15

seconds followed by an incubation period of a few minutes at room temperature (15 to 30°C). The samples were subsequently centrifuged at 12,000 × *g* for 15 minutes at 4°C in an Eppendorf® Microcentrifuge 5415 R.

3.2.3.1.3 RNA Precipitation

After centrifugation, three layers are apparent: an upper aqueous phase, an interphase and a bottom phenol chloroform phase. RNA in the upper aqueous phase was removed and transferred to a fresh tube. The RNA was precipitated from the aqueous phase by mixing with 375 µL of isopropyl alcohol to achieve a ratio of 0.5 mL of isopropyl alcohol per 1 mL of TRIzol® LS Reagent used for the initial homogenization. The samples were incubated at room temperature (15 to 25°C) for 10 minutes and subsequently centrifuged at no more than 12,000 × *g* for 10 minutes at 4°C.
Additionally, pure isopropyl alcohol was compared to a 1:1 mix of isopropyl alcohol and 1.2 M NaCl (Invitrogen, 2007; Watson, Personal Communication).

3.2.3.1.4 RNA Wash

The supernatant was poured off and the RNA pellet was washed once with 950 µL 75% (v/v) ethanol, adding at least 1 mL of 75% ethanol per 1 mL of TRIzol® LS Reagent used for the initial homogenization. The sample was mixed by vortexing briefly and centrifuged at no more than 7,500 × *g* for 5 minutes at 4°C.

3.2.3.1.5 Redissolving the RNA

The supernatant was poured off and the tubes returned to the centrifuge and spun down briefly to collect the last drops of ethanol. The tubes were then carefully aspirated and the RNA pellet left to air-dry for 5 minutes. The pellet was then dissolved in 40 µL of DEPC-treated water and incubated at 55-60°C for 10 minutes.

Standard protocols failed to yield detectable results and it was therefore adjusted to include some additional steps:

The additional steps resulted in four different RNA preparations that were compared (Table 3.3). In both situations, 4 µL of the extractions with 10 µL of formaldehyde loading buffer were run on ethidium bromide (0.5 µg/mL) containing 1.2 % (w/v) agarose gels in 0.5 × TBE.

Table 3.3: Summary of additional procedures in the TRIzol® LS extraction of AHS viral RNA from ECE

Number	Treatment
1	• Undiluted yolk as initial sample • RNA precipitated with IPA only
2	• Undiluted yolk as initial sample • RNA precipitated with IPA and 1.2 M NaCl
3	• 1:1 yolk : water as initial sample • RNA precipitated with IPA only
4	• 1:1 yolk : water as initial sample • RNA precipitated with IPA and 1.2 M NaCl

3.2.3.2 Extraction from cell culture

3.2.3.2.1 1. Homogenisation

1 mL aliquots for each serotype and control were thawed and transferred to a 15 mL Falcon™ tube (BD Biosciences, San Jose, USA). 3 mL of TRIzol® LS was added to achieve a 1:3 ratio of sample to reagent. The solution was homogenised by pipetting several times.

3.2.3.2.2 Phase Separation

To achieve the required phase separation, the homogenised samples were incubated at room temperature (15-25°C) for at least 60 minutes. 800 µL of chloroform was added and shaken vigorously by hand for 15 seconds. The shaken samples were incubated again at room temperature for 10-15 minutes and centrifuged at 12,000 × *g* for 15 mins at 4°C in a Beckman-Coulter Avanti® J-26XP centrifuge (JA-10 rotor).

3.2.3.2.3 RNA Precipitation

The solution separated into a lower red-pink phase, a white opaque interphase and an upper clear aqueous phase. RNA remains exclusively in the aqueous phase (Invitrogen, 2007). The upper aqueous phase was transferred to a new tube containing 750 µL of a 1.2 M sodium chloride/0.8 M sodium citrate sterile solution and 750 µL of ice-cold isopropyl alcohol. The tubes were inverted several times and incubated at room temperature for 10-15 minutes followed by a centrifugation at 12,000 × *g* for 10 minutes at 4°C.

3.2.3.2.4 RNA Wash

The resultant supernatant was discarded and the translucent pellet was washed by adding 4 mL of 75% ethanol in DEPC-treated water. The tubes were vortexed for 30 seconds and centrifuged at 7,500 × *g* for 5 minutes at 4°C. The supernatant was discarded and the pellet and remaining ethanol was aspirated and transferred to a 1.5 mL micro-centrifuge tube. The tubes were centrifuged at 7,500 × *g* for 5 minutes at 4°C in an Eppendorf® Microcentrifuge 5415 R. The remaining ethanol was carefully aspirated and the pellet air-dried for 5 minutes.

3.2.3.2.5 Redissolving the RNA

The pellet was subsequently re-suspended in 20 µL of DEPC-treated water and incubated at 55-60°C for 10 minutes. Absorbance readings were taken on the suspension after extraction with TRIzol® using a Thermo Scientific NanoDrop 1000.

3.3 Results

3.3.1 ECE

The standard RNA extraction protocol recommended by the TRIzol® manual revealed no results, indicating that no viral RNA was extracted from the harvested yolks.

The amended protocol (as indicated by italics above) resulted in the successful TRIzol® extraction of RNA from ECE yolk (Figure 3.2). Undiluted egg yolks and the lack of NaCl in the precipitation step (Treatments 1 and 3) produced a feint smear towards the bottom of the gel indicating that the addition of NaCl may enhance the precipitation of

low molecular weight RNA. The products seen in Figure 3.2 are confirmed to be RNA by the treatment of the samples with an RNase (Figure 3.3).

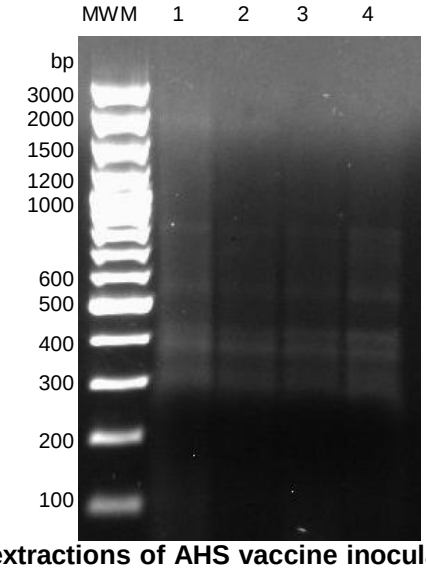

Figure 3.2: TRIzol® LS RNA extractions of AHS vaccine inoculated embryonated chicken egg yolks using four different treatments. Extractions were performed on 48 hour post inoculation egg yolks inoculated with 1:2 dilution of AHS vaccine vial 1 using the additional and modified procedures. 2 µL sample + 8 µL formaldehyde-containing loading buffer on a 1.2 % (w/v) agarose gel containing 1 µg/µL of ethidium bromide in 0.5× TBE.
MWM: Fermentas GeneRuler™ 100 bp Plus DNA ladder. Lane 1: Undiluted yolk, isopropyl alcohol precipitation of RNA. Lane 2: Undiluted yolk, isopropyl alcohol and NaCl precipitation of RNA. Lane 3: Diluted yolk, isopropyl alcohol precipitation of RNA. Lane 4: Diluted yolk, isopropyl alcohol and NaCl precipitation of RNA.

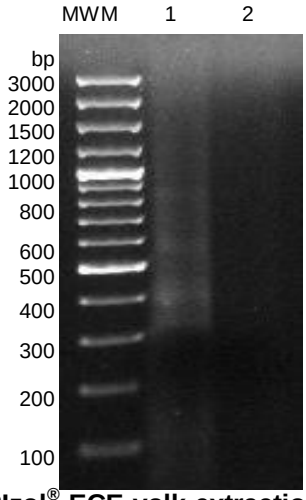

Figure 3.3: RNase treatment of TRIzol® ECE yolk extractions. TRIzol extracted RNA from AHS vaccine vial 1 1:2 dilution inoculated egg yolk showing before and after RNase treatments. 2 µL sample + 8 µL formaldehyde-containing loading buffer on a 1.2 % (w/v) agarose gel containing 1 µg/µL of ethidium bromide.
MWM: Fermentas GeneRuler™ 100 bp Plus DNA ladder. Lane 1: RNA before the addition of RNase. Lane 2: RNA sample after the addition of RNase.

3.3.2 Vero cell culture

The nine strains of AHSV were each inoculated into monolayers of Vero cells. The cytopathic effects seen were a general rounding of the cells and lifting off the surface of the flask. This was visible in all of the inoculations. An example is shown in Figure 3.4

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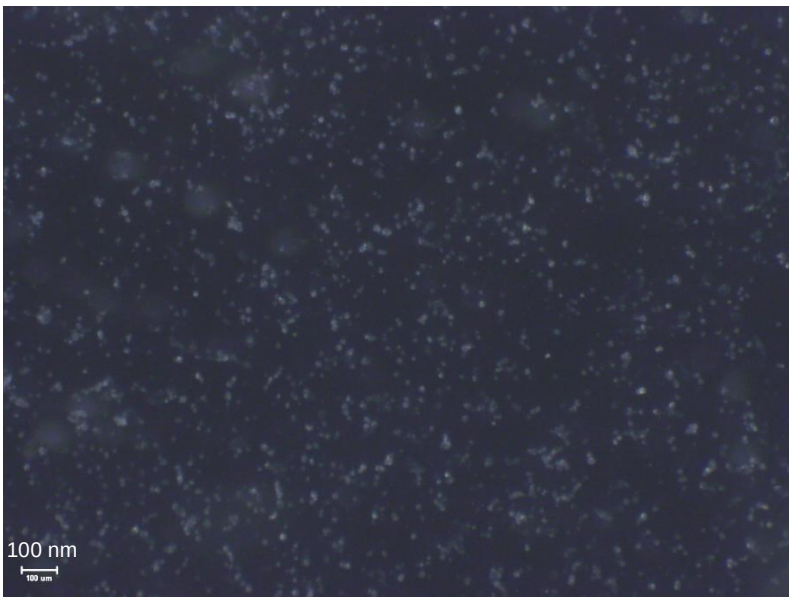


Figure 3.4: AHS inoculated (Serotype 7) Vero cell culture 84 hours post-inoculation. Rounded cells have altered refractory properties and appear opaque.

3.4 Discussion

Two propagation protocols for the AHS virus were evaluated for their relative merits. The relatively antiquated ECE protocol required less sophisticated equipment, but was laborious and unpredictable. It was compared with a more contemporary cell culture system that requires sophisticated equipment, but is more controlled.

Zero mortality in the chicken embryo's post inoculation is an indication of the avirulence of the AHS vaccine vial 1 and indicates that the vaccine virus can safely be grown in chicken embryos. To our knowledge, this is the first report of the inoculation of embryonated chicken eggs with a modern, live attenuated vaccine. No literature exists outside of the 1950's and 1960's that refers to growing the modern cell-cultured live attenuated vaccine virus in ECE's.

The standard procedure offered by TRIzol® manufacturers for the extraction of RNA from biological material produced no product from the inoculated ECE yolks. TRIzol®

has established itself as an industry standard commercial reagent based on the acid guanidinium thiocyanate-phenol-chloroform nucleic acid extraction procedure (Chomczynski & Sacchi, 1987). In a direct comparison between TRIzol® and a column-based technology, Rodriguez-Sanchez *et al.* (2008a), found that TRIzol® yielded consistently more RNA.

Egg yolk is a highly viscous substance and contains many other components and extracellular material, which may inhibit the one or more of the steps involved in the extraction procedure. A second extraction procedure was devised which contained additional steps to counter the nature of the yolk sample. Firstly, the yolk was diluted with DEPC-treated water in an effort to reduce the viscosity. Secondly, after addition of the required amount of TRIzol®, the mixture was homogenised with a 21G needle that exerted greater shearing forces on the cells and generated a homogenous solution. The third additional step was to centrifuge the solution before phase separation such that a greater amount of the insoluble material could be removed increasing the ratio of TRIzol® to the cells. After this centrifugation, four layers resulted: a top white, viscous phase; a middle pink phase; a white interphase and a bottom dark pink phase. The top layer was reasoned to contain most of the insoluble components due to its viscous nature and thus the bottom three layers were removed by aspiration and transferred to a new tube and vortexed. The fourth additional step involved creating a 1:1 dilution of isopropyl alcohol and 1.2 M NaCl such that the salt could augment the precipitation of RNA (Table 3.3).

No RNA was successfully extracted from the egg yolk using the standard protocol. Egg yolk contains a number of components that may seriously interfere with the action of TRIzol®. The amendment of the TRIzol® protocol (Table 3.3) resulted in RNA extractions (Figure 3.2). RNase successfully digested RNA in the egg yolk samples (Figure 3.3) to confirm the presence of RNA.

Needle homogenisation and extra centrifugation improved the product of the RNA extraction (Figure 3.2). NaCl may, however, result in low molecular weight RNA being extracted. (Figure 3.2). The clarity of the gel bands is improved in lanes 2 and 4 where IPA and NaCl were used in the precipitation step.

The AHS virus has been cultured in Vero cells in a number of studies and the protocol is fairly consistent and successful (Paweska *et al.*, 2003; OIE, 2004a; Koekemoer, 2008; Quan *et al.*, 2008; Rodriguez-Sanchez *et al.*, 2008a; von Teichman & Smit, 2008; Fernández-Pinero *et al.*, 2009; Guthrie *et al.*, 2009; Paweska, *Personal Communication*). The cultures were ready for harvesting between five and seven days post inoculation. Between two and eight days (OIE, 2004a) and between two and four days (Paweska *et al.*, 2003) have been reported. TRIzol® LS Regent (Invitrogen, Walkersville, USA) is a phenol and guanidine isothiocynate solution based on the RNA isolation step developed by Chomczynski and Sacchi (1987) (Invitrogen, 2007). A 2-step RT-PCR protocol was followed such that a stock of cDNA could be kept. The standard protocols of the Quantace SensiMix™ 2-step kit were successfully applied to the extraction of AHS viral RNA. Confirmation of the correct size amplicon was obtained by 180-182 bp bands visualised on a 1.2% (w/v) agarose gel containing 1 µg/µL of ethidium bromide in 0.5 × TBE. PCR products were sequenced by Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). The control and NTC did not produce any product.

3.5 Conclusion

AHS virus can successfully be grown using Vero cell culture and the RNA extracted using TRIzol® LS Reagent. The African horse sickness vaccine virus was also successfully propagated in embryonated chicken eggs (ECE) via the yolk sac route. Although the intravascular route is recommended for increased yields (Boorman *et al.*, 1975), it requires a high level of expertise. ECE represents an alternative to cell culture techniques but can prove to be complicated due to the nature of the yolk and the extraction method.

Chapter 4: BIOINFORMATIC ANALYSIS AND PRIMER DESIGN

4.1 Introduction

The African horse sickness virus genome is composed of ten common double-stranded RNA segments (Grubman & Lewis, 1992). Only nine serotypes have been discovered (Howell, 1962), with two of the segments responsible for the genetic diversity amongst the serotypes, namely Segment 2 and 10 (Roy *et al.*, 1994; Venter *et al.*, 2000).

In order to develop an assay that is cost- and time-efficient, the primary goal is to find suitable regions across all nine serotypes that are flanked by nearly identical regions, but have divergent sequences in between (Figure 4.1)

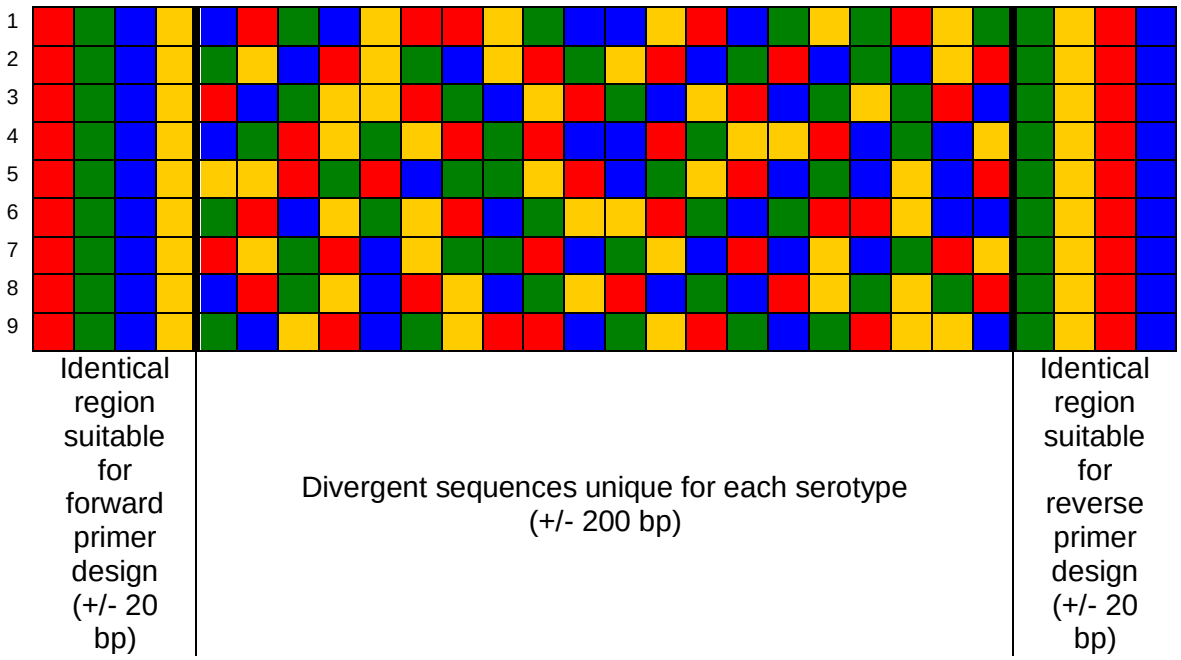


Figure 4.1: Schematic representation showing the ideal primer design across multiple sequences, such as that sought for all of the nine African horse sickness virus serotypes to develop a serotyping assay.

Ideally, a single primer pair is used in the final assay. Using more than one primer pair in a reaction requires multiple reactions or multiplex PCR and its complications and inevitably increases cost factors (Henegariu *et al.*, 1997; Elnifro *et al.*, 2000).

The aim, therefore, when designing primers for this assay, was to select a region that was flanked by conserved regions from which primers could be designed, and that contains an amplicon region that was divergent among the serotypes, so that they would produce significantly different melt curves in the HRM analysis.

The optimal design of a primer will depend on the region to be amplified and the flanking regions. However, there exist some common recommendations and guidelines. These are outlined as follows (Dieffenbach *et al.*, 1993; Rybicki, 2001):

- The optimum melting temperature (T_m) should be between 55°C and 65°C, although it can be substantially lower or higher, but with consequences for specificity and sensitivity. In addition, it must be noted that there are two primers per reaction and that the T_m's should be reasonably close to each other to aid efficient amplification.
- The design of primers should avoid any dimersation abilities, *viz*. the primers should not contain complementary regions between themselves such that they bind to each other.
- Individual primers should not contain any complementary regions within themselves. This may cause the primers to bind to themselves and form ‘hairpin’ structures.
- The target DNA sequence should also be checked to ensure that the primer could only bind in one position.
- The primers should be between 17-28 nucleotide bases in length
- The composition of the guanidine and cytosine (GC) bases should be 50-60%
- The 3’ end of the primer should end in a guanidine or cytosine. Guanidine and cytosine are joined by three hydrogen bonds and are therefore stronger than the double hydrogen bonds of an adenine-thymine pairing.
- A series of three or more Gs or Cs at the 3'-ends of primers should be avoided. As they are more stable, they may mis-prime at G or C-rich sequences

- Complementary 3'-ends of primer pairs should be avoided. This may result in primer dimers forming

Many software programs are freely available to design suitable primer pairs. Licensed software packages are also available. One of the most commonly used freeware programs is *Primer3* (Rozen & Skaletsky, 2000). *Primer3* is the continuation of a software development started in 1991 at the Whitehead Institute and funded by the Howard Hughes Medical Institute and the National Institutes of Health. *Primer3* automatically takes into account the above factors to locate the most suitable regions for primer binding.

Primer3 is designed to pick a single primer pair for one sequence at a time. As mentioned above, the assay depends on the ability to find primers that are common to all nine serotypes. Recognising this inadequacy of *Primer3*, Gadberry *et al.* (2005) designed a web-based application 'to find conserved PCR primers across multiple species'. In essence, the program, called *Primaclade*[2] runs a *Primer3* analysis on each sequence entered and then merges the results.

AHSV sequences are readily available on *GenBank. GenBank* has developed into one of the most valuable tools for bioinformatics. Over 79 million sequences are available for over 260,000 organisms. Individual laboratories from all over the world are responsible for the majority of sequences submitted (Benson *et al.*, 2009). As such, a huge number of sequences are available for identical genomes and can be taken into account when designing primers.

The success of the proposed assay is entirely dependant on the design of a primer pair and the characteristics of the amplicon. The analysis of Segment 2 and 10 of the African horse sickness virus genome begins with the collection and collation of all available sequences through *GenBank* (Burks *et al.*, 1985; Benson *et al.*, 2009). A variety of selected sequences are then aligned using the *Clustal* (Chenna *et al.*, 2003; Larkin *et al.*, 2007) program and a cladogram drawn using *TreeView* (Page, 1996) to reveal the extent of sequence divergence.

[2] http://www.umsl.edu/~biology/Kellogg/Primaclade.html

4.2 Materials and Methods

In all analyses, both Segment 2 and Segment 10 of the AHS viral genome were considered.

4.2.1 Sequence Retrieval

An analysis of any genomic sequence invariably begins with *GenBank*. *GenBank* is a service offered by the National Center for Biotechnology Information (NCBI) and hosted at http://www.ncbi.nlm.nih.gov. Searching the 'Nucleotide' database for "African horse sickness Segment 2 vp2", 45 results were recovered. Sequences that only contain the coding sequences or partial coding sequences were discarded. 16 sequences were found to be complete and contain the full-length genome for Segment 2. When a search was performed for 'African horse sickness Segment 10 NS3', 23 sequences were found and all were relevant for downstream analysis.

4.2.2 Sequence Alignment

A number of different programs have been designed that enable the alignment of multiple sequences using various algorithms. This allows one to compare the identity between different sequences. A freeware alignment software is known as *Clustal* and the most recent Microsoft® Windows® interface is known as *ClustalX2* (Larkin *et al.*, 2007). *Clustal* also allows one to produce various dendrograms based on the alignment algorithms (Chenna *et al.*, 2003). Sequences are downloaded from *GenBank* in FASTA format, loaded into *Clustal* and the alignment formed. Full alignments are given in Appendix 1 (sequence Segment 2) and Appendix 2 (sequence Segment 10). By way of example and ease of reference, the first 60 bases of AHSV Segment 2 from 16 full-length sequences are shown here in Figure 4.2.

```
Identifier                Sequences and alignment indices
AHSV8_DQ868775        GTTTAATTCATCATGGCGTTCGAGTTTGGCATATTACTAACTGAGAAAGTAGAAGGTGAT
AHSV8_AY163333        GTTTAATTCATCATGGCGTCGGAGTTTGGCATATTACTAACTGAGAAAGTAGAAGGTGAT
AHSV5_AY163331        GTTTATTTCATCATGGCTTCAGAGTTTGGCGTTCTGTTGACCGATAAAGTTGAAGGCGAT
AHSV6_DQ868774        GTTAAATTCACCATGGCTTCCGAATTTGGCATTTTGATTTGTGATAAATTAAAGGAAAAC
AHSV6_NC_005996       GTTAAATTCACCATGGCTTCCGAATTTGGCATTTTGATTTGTGATAAATTGAAGGAAAAT
AHSV9_DQ868776        GTTTAATTCACCATGGCGTTCGAGTTTGGAATACTTCAGACGGACAAAATTAGAGAGAAT
AHSV4_EU046574        GTTTAATTCACCATGGCGCCCGAGTTTGGAATATTGATGACAAATGAAAAATTTGACCCA
AHSV4_DQ868773        GTTTAATTCACCATGGCGTCCGAGTTTGGAATATTGATGACAAATGAAAAATTTGACCCA
AHSV4_D26570          GTTTAATTCACCATGGCGTCCGAGTTTGGAATATTGTTGACAGATGAAAAATTTGACCCG
AHSV1_FJ011108        GTTTATTTCAGCATGGCGTCTGAATTTGGAATTCTATTGACCGAGAGAATCTTTGACGAA
AHSV1_AY163329        GTTTATTTCAGCATGGCGTCTGAATTTGGAATTCTATTGACCGAGAGAATCTTTGACGAA
AHSV2_AY163332        GTTTATTTCAGCATGGCGTCTGAATTTGGAATACTTTTCACCGAAAAGATCTATGACCAA
AHSV1_Z26316          GTTTAATTCACCATGGCTTCGGAATTCGGGATCCTATTGACAAATCAAATATATGATCAA
AHSV3_U01832          GTTTAATTCACCATGGCTTCGGAATTCGGGATCCTATTGACAAATCAAATATATGATCAA
AHSV3_DQ868772        GTTTAATTCACCATGGCTTCGGAATTCGGGATCTTATTGACAATTCAAATATATGATCAA
AHSV7_AY163330        GTTTAATTCACTATGGCTTCTGAGTTTGGAATTTTGTACACTGATCAGATCTACGAACAA
                      *** * ****  *****    ** ** **  *  *                  *
```

Figure 4.2: *ClustalX2* alignment of 16 sequences of African horse sickness virus Segment 2 taken from *GenBank*.

The asterisks below the sequences (alignment indices) represent identical bases across the alignment. The first column contains a unique identifier assigned to the sequences. The number after AHSV represents the serotype that that particular sequence comes from and the code following the underscore are the unique accession numbers assigned to each submitted sequence on the *GenBank* database.

It is important to note that the entire sequence alignment must be studied for a suitable region to be found. Despite there only being nine known serotypes, the sequences found on *GenBank* all represent actual sequenced data of the viral genome. As such, it is imperative that all known sequences are used to establish an alignment as opposed to just nine (one of each serotype) in order that the most accurate data is used concerning the most up-to-date and historical genetic state of the viral genome. In terms of published literature, the genomic comparison of more than one serotype is rarely performed due to the lack of research surrounding genetic/molecular serotyping.

4.2.3 Dendrogram Visualisation

A dendrogram enables one to infer any sequence identities and the relationships between sequences based on their relative identities and differences. Full-length alignments of both Segment 2 and 10 were visualised in a dendrogram using *TreeView.* Following the alignment and selection of suitable primer pair sites, the proposed amplicon (containing the primer sites) was subjected to a secondary

alignment. This alignment was then visualised in a dendrogram for relative sequence identities and relationships. The separation of sequences on a dendrogram is likely to give an indication of the relative melt profiles after HRM analysis. Dendrograms of the full-length sequence were compared such that the ability to separate sequences on a short amplicon versus the entire full-length segment could be assessed.

4.2.4 Primer Design

Both segments were scrupulously analysed for potential primer sites. Using the *Clustal*X alignment files, ideal sites would be regions where there are approximately 20 asterisks in a row flanking a region of approximately 200 base pairs where the asterisks are very erratic across all 16 sequences. In addition to the manual, visual inspection of the alignment for suitable primer regions, the alignment files were submitted to *Primaclade.* Simply stated, *Primaclade* runs *Primer3* on each sequence in the alignment and produces a consensus sequence. From this consensus sequence, possible primer sites are shown. The *Primaclade* results were compared to the manual, visually designed primer pairs.

4.2.5 BLAST

It is important to confirm the specificity of the chosen primer pair. Primer pairs that are not highly specific may lead to incorrect priming on either a different part of the segment or genome. Alternatively, it may be possible that they could prime to unrelated sequences from different organisms, such as the host cell in which they were cultured. To assess their specificity, the Basic Local Alignment Search Tool (BLAST) is used. As part of the NCBI's compendium of bioinformatic programs, it searches through the entire *GenBank* database for possible matches.

4.2.6 POLAND

POLAND is a program that calculates the thermal dissociation patterns of dsDNA and RNA, in essence, an *in silico* melt profile with resultant peaks (Steger, 1994). The program can be used to predict melt profiles in assay design. The 10-190 bp regions of Segment 10 were analysed by the POLAND program.

4.3 Results

TreeView (Page, 1996) is a freeware program that converts the alignment file from *Clustal* into a dendrogram. Figure 4.3 represents the dendrogram achieved after the alignment of full-length sequences of Segment 2 (Appendix 1).

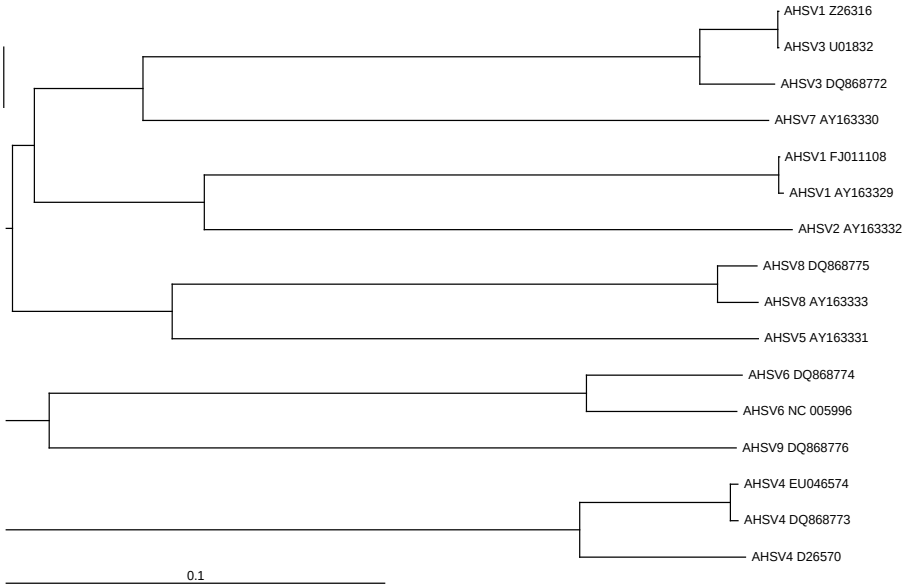


Figure 4.3: The dendrogram (*TreeView*) produced from the full-length sequences of Segment 2 of the African horse sickness virus genome. The bar represents 0.1 substitutions per site.

The dendrogram of Segment 2 reveals very little identity between the sequences and distinct groupings are not easily discerned. The alignment of the full length AHSV Segment 10 (Appendix 2) is shown as a dendrogram in Figure 4.4.

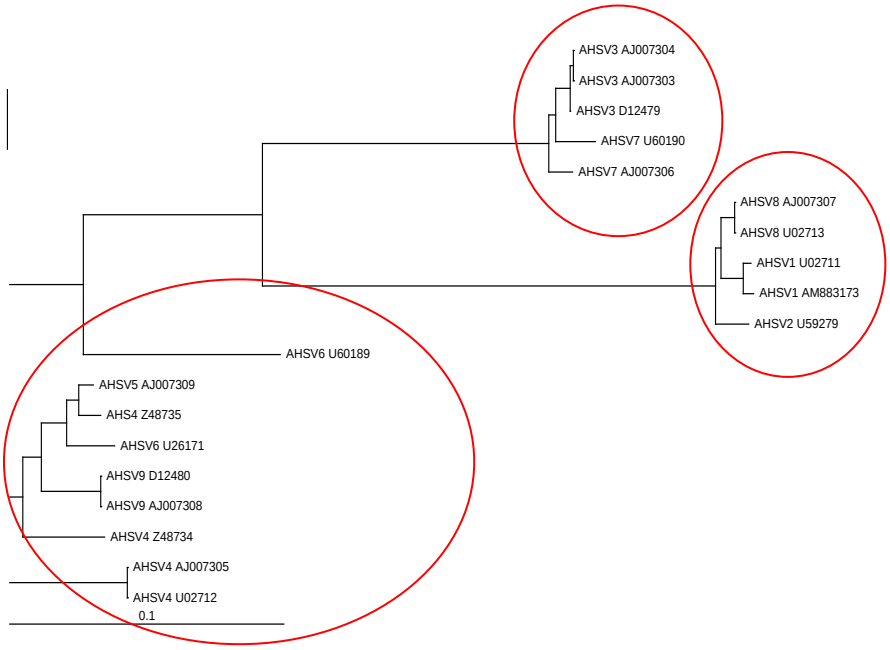


Figure 4.4: The dendrogram (*TreeView*) produced from the full-length sequences of Segment 10 of the African horse sickness virus genome. The bar represents 0.1 substitutions per site.

Three distinct groupings exist in the dendrogram in Figure 4.4 as shown by the red circles. Figure 4.5 displays the dendrogram achieved after the alignment of the 10-190 bp region of Segment 10. This was the only region that complied with the conditions of being a single primer pair across all nine serotypes. These groupings of the full-length Segment 10 (Figure 4.4) dendrogram and the 10-190 bp regions (Figure 4.5) coincide.

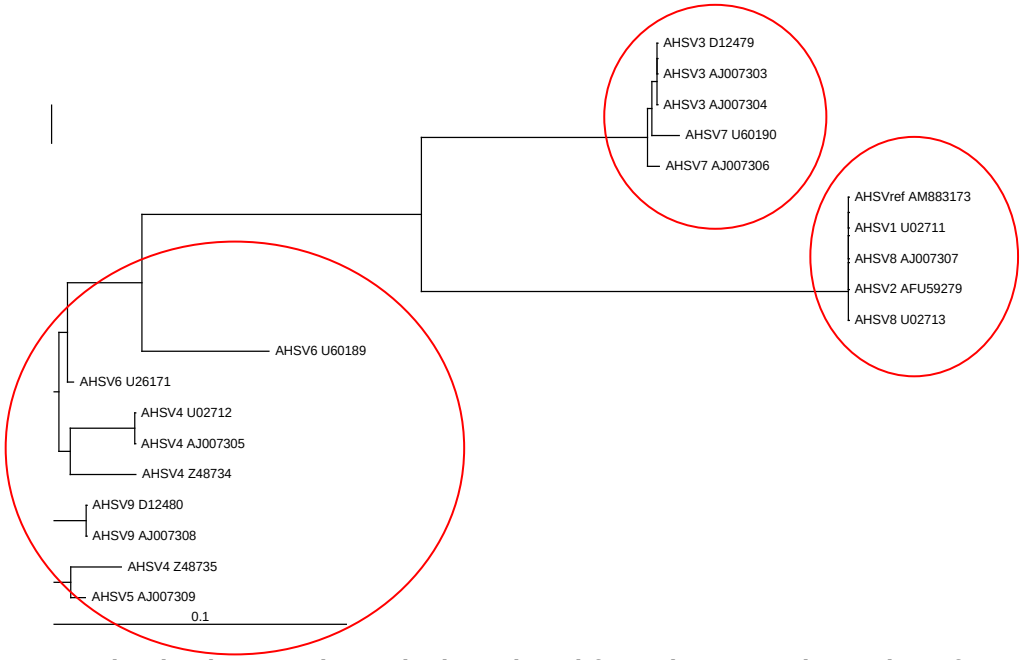


Figure 4.5: The dendrogram (*TreeView*) produced from the 10-190 bp region of Segment 10 of the African horse sickness virus genome amplified by the primer pair *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev*. The bar represents 0.1 substitutions per site.

Serotypes 1, 2 and 8 have an identical amplicon and will not be distinguishable using the 10-190 bp region of Segment 10. Divergence within serotype 3 is low, while Serotype 7 is closely related to serotype 3. Serotype 6 is segregated from the nodes of serotype 4 and serotype 9. Serotype 5 exists in a node with one of the serotype 4's. Three distinct groupings are easily identified, as shown by the circles.

The conditions for the selection of primers for the proposed assay were not met for Segment 2. Two primer sites on Segment 10, however, amplify a 180 bp region from position 10 to 190 across the 23 known sequences of the nine serotypes (Figure 4.6).

```
AHSV3_AJ007304          GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV3_D12479            GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV3_AJ007303          GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV7_U60190            GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV7_AJ007306          GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV4_U02712            GTTTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAATTATAGTATGCATAA
AHSV4_AJ007305          GTTTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAATTATAGTATGCATAA
AHSV4_Z48734            GTTTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAATTATAGTATGCATAA
AHSV4_Z48735            GTTTAAATTATCC-TTGTCATGAATCTAGCTACAATCGCCAAGAATTATAGCATGCATAA
AHSV5_AJ007309          GTTTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAATTATAGCATGCATAA
AHSV9_D12480            GTTTAA-TTATCCCTTGTCATGAATCTAGCTGCAATCGCCGAAAATTATAGTATGCATAA
AHSV9_AJ007308          GTTTAA-TTATCCCTTGTCATGAATCTAGCTGCAATCGCCGAAAATTATAGTATGCATAA
AHSV6_U26171            GTTTAA-TTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAATTATAGTATGCATAA
AHSV6_U60189            --TTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAACTACAGTATGCATAA
AHSVref_AM883173        GTTTAA-TTATCCCTTGTCATGAATCTTGCTAGCATCTCCCAAAGCTATATGTCACATAA
AHSV1_U02711            GTTTAAATTATCCCTTGTCATGAATCTTGCTAGCATCTCCCAAAGCTATATGTCACATAA
AHSV8_AJ007307          GTTTAAATTATCCCTTGTCATGAATCTTGCTAGCATCTCCCAAAGCTATATGTCACATAA
AHSV2_AFU59279          GTTTAAATTATCCCTTGTCATGAATCTTGCTAGCATCTCCCAAAGCTATATGTCACATAA
AHSV8_U02713            GTTTAAATTATCCCTTGTCATGAATCTTGCTAGCATCTCCCAAAGCTATATGTCACATAA
                          **** ****** ********* *** ***   *** ** * *  ** *     *****

AHSV3_AJ007304          TGGAAATCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV3_D12479            TGGAAATCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV3_AJ007303          TGGAAATCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV7_U60190            TGAAACTCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV7_AJ007306          TGGAAATCAGAGAGCAATTGTGCCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV4_U02712            TGGAGAGTCGGGGGCGATTGTCCCTTATGTGCCACCGCCATATAATTTCGCGAGCGCTCC
AHSV4_AJ007305          TGGAGAGTCGGGGGCGATTGTCCCTTATGTGCCACCGCCATATAATTTCGCGAGCGCTCC
AHSV4_Z48734            TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATGTTGCGAGTGCTCC
AHSV4_Z48735            TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV5_AJ007309          TGGAGAGTCGGGGACGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV9_D12480            TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV9_AJ007308          TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV6_U26171            TGGAGAGTCGGAGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV6_U60189            TGGAGAGCAAGAGGCGATCGTCCCATATGTGCCGCCACCATACAATTTCGCAAGTGCTCC
AHSVref_AM883173        TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
AHSV1_U02711            TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
AHSV8_AJ007307          TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
AHSV2_AFU59279          TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
AHSV8_U02713            TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
                        **           * ** ** ** **  * ** ** ** **       * *  *****
```

```
AHSV3_AJ007304          GACGCTTGGTGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAATCAAGC
AHSV3_D12479            GACGCTTGGTGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAATCAAGC
AHSV3_AJ007303          GACGCTTGGTGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAATCAAGC
AHSV7_U60190            GACGCTTGGTGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAATCAAGC
AHSV7_AJ007306          GACGCTTGGTGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAATCAAGC
AHSV4_U02712            GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV4_AJ007305          GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV4_Z48734            GACGTTTTCTCAGGGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV4_Z48735            GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV5_AJ007309          GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV9_D12480            GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV9_AJ007308          GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV6_U26171            GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV6_U60189            GGCGCTTCCTCAGCGTACAAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAGGC
AHSVref_AM883173        GGCGCTTGCTGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGATACTTAACCAAGC
AHSV1_U02711            GGCGCTTGCTGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGATACTTAACCAAGC
AHSV8_AJ007307          GGCGCTTGCTGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGATACTTAACCAAGC
AHSV2_AFU59279          GGCGCTTGCTGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGATACTTAACCAAGC
AHSV8_U02713            GGCGCTTGCTGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGATACTTAACCAAGC
                        * ** **  *       *  ** ******** ** ****************** ** **

AHSV3_AJ007304          CATGTCAAGTACAACTGGTGCAAGTCGGGC----
AHSV3_D12479            CATGTCAAGTACAACTGGTGCAAGTCGGGC----
AHSV3_AJ007303          CATGTCAAGTACAACTGGTGCAAGTGGGGC----
AHSV7_U60190            CATGTCAAGTACAACTGGTGCAAGTGGGGC----
AHSV7_AJ007306          CATGTCAAGTACAACTGGTGCGAGTGGGGC----
AHSV4_U02712            CATGTCAAGTACAACTGGTGCGAGTGGGGC----
AHSV4_AJ007305          CATGTCAAGTACAACTGGTGCGAGTGGGGC----
AHSV4_Z48734            CATGTCAAATACAACTGGTGCGAGTGGGGC----
AHSV4_Z48735            CATGTCAAGTACAACTGGTGCGAGTGGGGCG---
AHSV5_AJ007309          CATGTCAAGTACAACTGGTGCGAGTGGGGC----
AHSV9_D12480            CATGTCAAGTACAACTGGTGCGAGTGGGGCG---
AHSV9_AJ007308          CATGTCAAGTACAACTGGTGCGAGTGGGGCG---
AHSV6_U26171            CATGTCAAGTACAACTGGTGCGAGTGGGGCG---
AHSV6_U60189            CATGTCAAGTACAACTGGTGCGAGTGGGGCAC--
AHSVref_AM883173        AATGTCAAGTTCAGCTGGTGCGAGCGGAGCACTT
AHSV1_U02711            AATGTCAAGTTCAGCTGGTGCGAGCGGAGCACT-
AHSV8_AJ007307          AATGTCAAGTTCAGCTGGTGCGAGTGGCGCACT-
AHSV2_AFU59279          AATGTCAAGTTCAGCTGGTGCGAGTGGCGCACT-
AHSV8_U02713            AATGTCAAGTTCAGCTGGTGCGAGTGGCGCACT-
                         ******* * ** ******* **  * **
```

Figure 4.6: The selection of two primer sites for the African horse sickness virus Segment 10 across the *Clustal* alignment of the 19 selected sequences located on *GenBank*. The primer sites are indicated in red and amplify a 180 bp product from position 10 to 190.

The primers, therefore, are as follows and amplify a 180 bp region from position 10-190 (Table 4.1):

Table 4.1: The selected primers and calculated properties to amplify a 180 bp product from position 10-190 of the African horse sickness virus Segment 10 (Bioneer, Daejeon, Korea)

Primer	*Sequence*	*Length*	T_m
AHS Seg10 10-190Fwd	ATCCCTTGTCATGARTCTWGCT	22	59.3°C
AHS Seg10 10-190Rev	CTTGACATKGCTTGRTTAAGTATCC	25	57.5°C

The annealing temperatures (T_m) of each primer were calculated using the OliCalc function in *pDRAW32*. Bioneer calculated the T_m's of the primer pair but did not provide any conditions or method of calculation for verification. The degeneracies of the primer pair require some minor adjustments to the primer pair in order to calculate an accurate T_m. The 'bases' R, W and K may represent one of two bases – R: G/A; W: A/T and K: G/T. Since A and T contribute to a lower melting point, they were substituted into the primer in order to calculate the T_m. The PCR reaction conditions also affect the T_m, and, as such, the following conditions were defined according to the Quantace SensiMix™ 2 step kit protocol: Primers = 200 nM; NaCl + KCl = 50 mM; $MgCl_2$ = 3 mM; glycerol = 1 % (w/v); formamide = 0 % (w/v) and DMSO = 0 % (w/v).

The relative position of the primers on Segment 10 of the AHSV genome are shown in Figure 4.7 according to the program pDRAW32 (Tippmann, 2004).

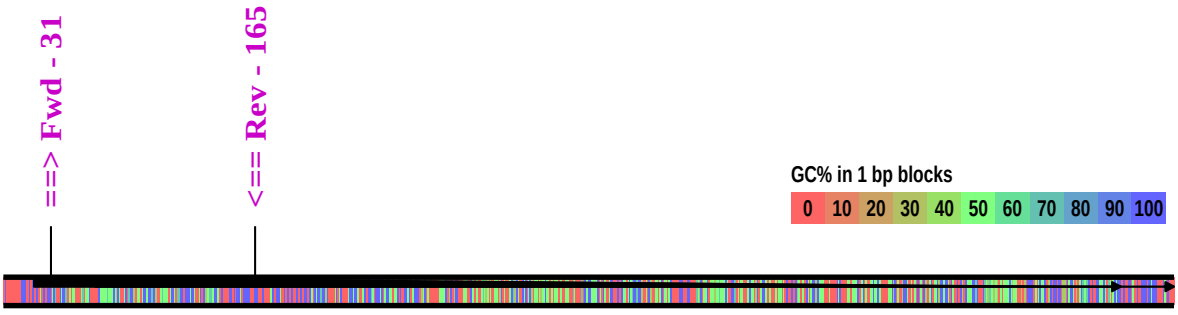
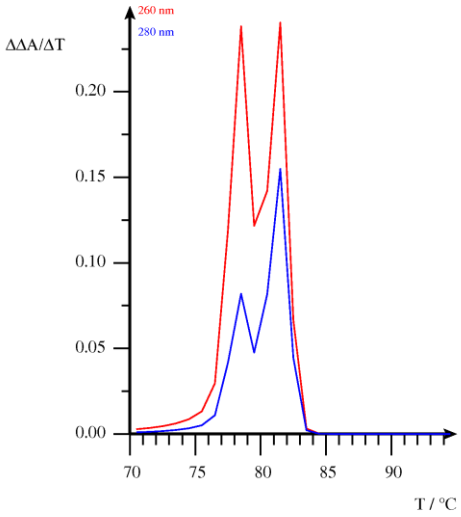


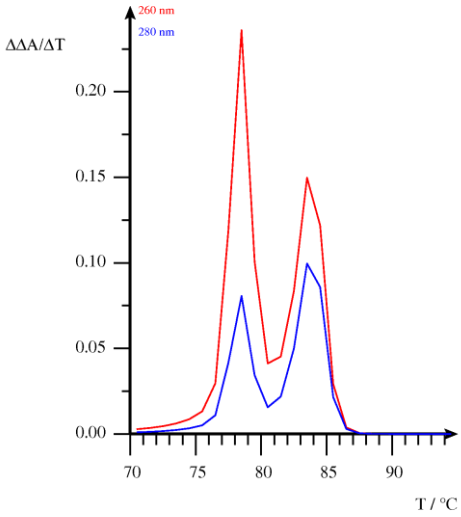
Figure 4.7: Relative positions of *AHS Seg10 10-190Fwd* (Fwd) and *AHS Seg10 10-190Rev* (Rev) with reference to a consensus sequence of 19 sequences of Segment 10 of the African horse sickness virus genome as defined by pDraw32. The numbers represent the position of each primer towards the centre of the amplicon.

Therefore, the 180 bp primer selection (*AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev*) is theoretically ensured of discriminating between the serotypes 9, 4 and 6; 3 and 7; and 1, 2 and 8. The significance of the primer selection in amplifying appropriate regions of the AHSV genome is that any field/vaccine strains of AHSV in serum will be identified to serotype.

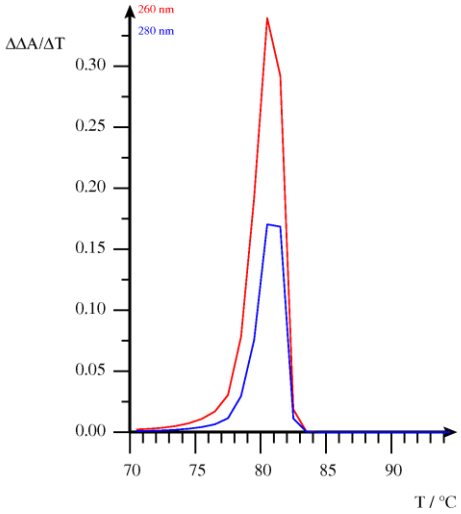
A *GenBank* sequence representative of each serotype was used to determine the simulated melt profiles using POLAND (Steger, 1994) using the 10-190 bp region of Segment 10 (Figure 4.8).



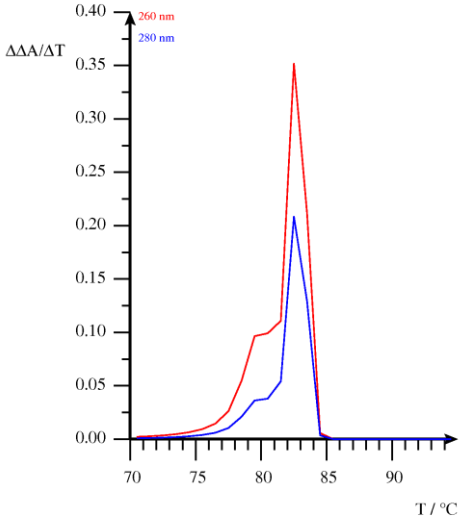
AHSV1_U02711



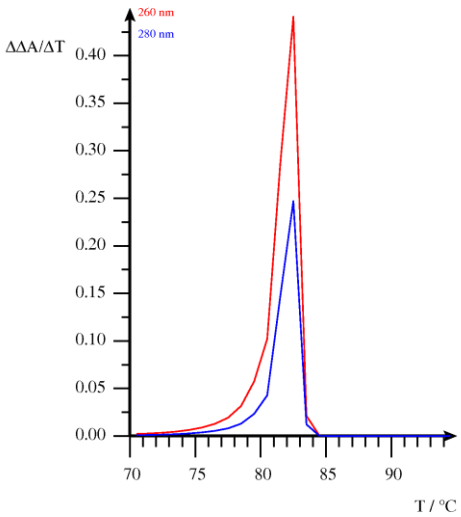
AHSV2_AFU59279



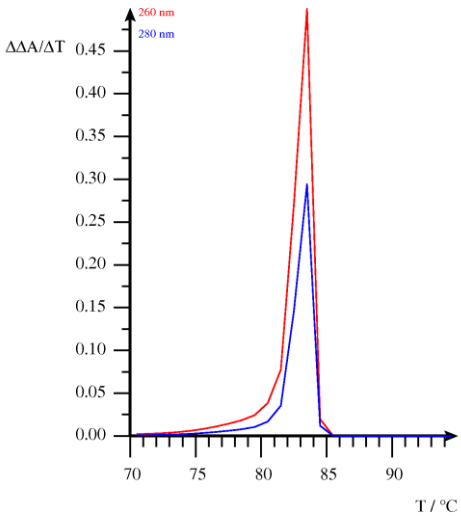
AHSV3_D12479



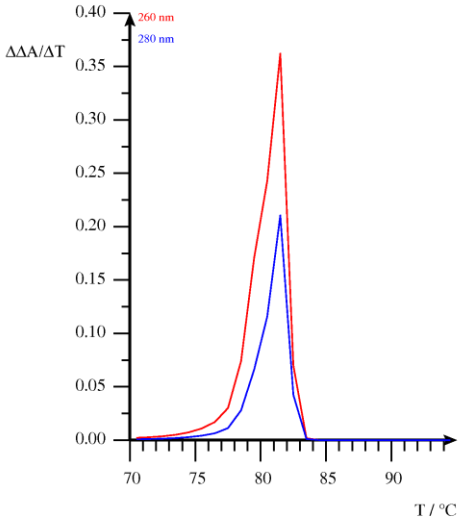
AHSV4_AJ007305



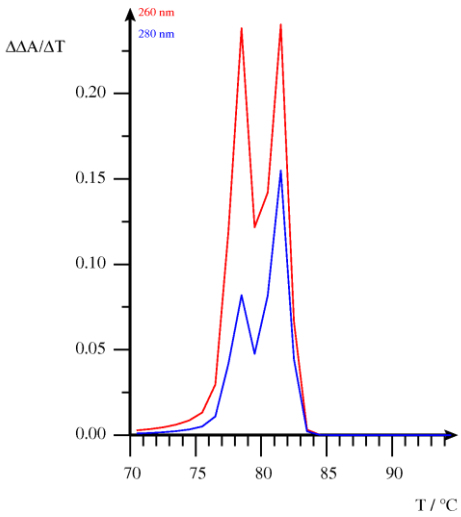
AHSV5_AJ007309



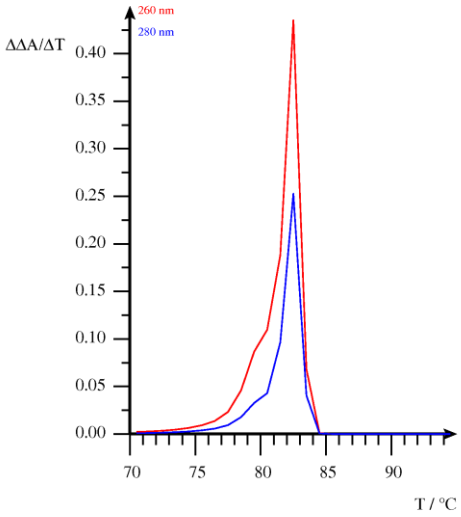
AHSV6_U60189



AHSV7_AJ007306



AHSV8_AJ007307



AHSV9_D12480

Figure 4.8: Simulated melt profiles of the 10-190 bp regions of Segment 10 using POLAND. The serotype and *GenBank* accession number from which the amplicon was selected are found under each graph.
The red and blue lines use the predicted absorbance readings at 260 nm and 280 nm respectively to draw the graph and represent the change in absorbance over temperature.

4.4 Discussion

Previous phylogenetic studies of the African horse sickness virus have been rather limited due to the lack of sequences available (de Sá *et al.*, 1994) or the fact that only one sequence per serotype may have been considered (Sailleau *et al.*, 2000). Recent research on serotype determination using Segment 2 and Segment 10 has greatly increased the database of AHSV serotypes in *GenBank* and consequently improved the integrity of primer selection.

The first segment to be considered when considering serotype specific tests would be Segment 2 as it is the primary genome segment responsible for serotype specificity (Roy *et al.*, 1994). However, upon visual and *in silico* analysis of the alignment (Appendix 1), no suitable region could be found for primer design. This was confirmed by the dendrogram analysis that revealed the large divergence in the serotypes (Figure 4.3). In addition, Segment 2 is approximately 3205 bp in length, while Segment 10 is approximately 756 bp in length (Mertens *et al.*, 2006). In Figures 4.3 to 4.5, the scale bar represents 0.1 nucleotide substitutions per site. In other words, following and measuring the horizontal branches from one entry to another, a distance ten times the length of the bar would represent one substitution. Taking into account the length of the horizontal branches, Segment 10 is clearly more conserved than Segment 2. Segment 10 presented greater opportunities in terms of the alignment and possible primer regions and was represented to a much greater degree on *GenBank*.

Variation in sequences is due to single or multiple base pair substitutions or gaps in one or more sequences. For primer selection, conserved regions in more or less identical regions should be aligned such that they are reasonably and suitably spaced by variable regions. The *Clustal* programs were developed in 1988 by Des Higgins and continue to improve (Chenna *et al.*, 2003). As explained above, asterisks (*) represent identical, convergent regions and dashes (-) represent areas where gaps have been introduced in order to create a better alignment. In the alignment of Segment 2, no suitable regions could be found without introducing a large number of degenerate bases in the primer sequence. (Degenerate bases are bases in the primer sequence that can be interchanged in order to complete the primer.) Segment 10 provided suitable primer regions. The selected Segment 10 primers contained minimal degenerate bases (where R represents a G or A; W represents an A or T and K

represents a G or T.) The length is ideal (Dieffenbach *et al.*, 1993) and the theoretical T_m is within one degree of the other. However, different programs will give different T_m's based on the particular method and calculation that is used. As a result, *AHS Seg10 10-190Fwd* has a T_m of 54°C according to Bioneer and 53.5°C when using the program pDRAW32 and ranges from 53.5°C to 58.2°C when individual bases are substituted for the degenerate ones calculated using the T_m calculator on pDRAW32. *AHS Seg10 10-190Rev* has a T_m of 55.1°C according to Bioneer and 43°C when using the program pDRAW32 and ranges from 50.4°C to 55.8°C when individual bases are substituted for the degenerate ones calculated using the T_m calculator on pDRAW32. Primer selection using *Clustal* was verified using *Primaclade*.

In Figure 4.8, the use of POLAND to predict the melt profiles showed that serotypes 1, 2 and 8, with a common melt profile, were also found in the same grouping in the dendrogram (Figure 4.5). The other groupings can also be seen with similarities between serotypes 3 and 7 and also serotypes 4, 5, 6, and 9.

Despite the fact that the best possible primer selection for Segment 10 might not be able to differentiate the nine serotypes exclusively, melt curves that are uniquely associated with each node would prove the ability of HRM to differentiate viral RNA that belongs to particular dendrogram groupings from clinical samples. Further bioinformatic analysis will investigate the use of probes or two different primer pairs to differentiate all nine serotypes uniquely and conclusively in the context of High Resolution Melt analysis.

4.5 Conclusion

The combination of *GenBank*, *Clustal*X2, *Primaclade*, *TreeView* and BLAST represent an ideal suite of programs for bioinformatic analysis and primer design. In addition, they are all freely available. Following the inadequacies of Segment 2 for the assay to be developed, Segment 10 represented another option. The primers *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev* were chosen to amplify the 10-190 bp region of Segment 10 of the AHSV genome for all nine serotypes. Degeneracies were unavoidably present, however. Although the dendrogram analysis reveals the inadequacies for this primer pair as far as the objectives of the assay are concerned, they represent the preliminary investigations for proof of concept to be established.

Chapter 5: AMPLIFICATION AND ANALYSIS OF EXTRACTED AHS VIRAL RNA

5.1 Introduction

The development of a rapid assay for the AHS virus is largely dependent on the characteristics of the techniques available and their inherent rapidity. Molecular diagnostics based on nucleic acid amplification, such as RT-PCR, represent the most modern, commonly used procedures to achieve rapid assays for the detection of pathogens. Indeed, infectious disease diagnosis has been revolutionised by PCR-based molecular detection systems (Eaton & White, 2004).

PCR has additional advantages in that it can detect the virus during the early stages of viraemia and in samples that have been poorly preserved, unlike viral isolation techniques. PCR achieves results in less than 24 hours as opposed to the more traditional viral isolation methods where it may take five days and sometimes up to 15 days for a positive result in less virulent strains (Stone-Marschat *et al.*, 1994; Sailleau *et al.*, 1997).

The significance of this research lies within the advantages of PCR combined with High Resolution Melt analysis (HRMA) for detecting the AHS virus in the blood or other biological sample of infected equids. Noteworthy advantages exist in using PCR and HRMA in clinical situations where conventional microbiology is inadequate, time-consuming or labour-intensive (e.g. cell culture), or difficult and hazardous (Abdalla *et al.*, 2002). The rapid nature of PCR also has important consequences for limiting the spread of highly contagious pathogens in an epidemic.

High resolution melting (HRM) was introduced in 2002 through the collaborative efforts of academia (University of Utah) and industry (Idaho Technology) (Reed *et al.*, 2007). A variety of methods have previously been developed to detect DNA sequence variation of PCR products. However, these involve extra processing and separation steps subsequent to the PCR run, include additional apparatuses and are time-consuming. Gundry *et al*. (2003) described the ability of melt temperatures to

distinguish unique variants in a homogenous, closed tube procedure performed automatically after PCR. HRM requires normal PCR reagents, a fluorescing dsDNA-binding dye and a short period of closed-tube, post-PCR analysis (Reed & Wittwer, 2004). Reed *et al.* (2007) considers HRM the simplest method of determining sequence variation and recognises its increasing popularity.

As discussed in Chapter 4, primers were selected according to their ability to amplify a portion of Segment 10 that may reveal HRM curves suitable for downstream analysis, after performing a standard 2-step RT-PCR.

5.2 Materials and Methods

5.2.1 Real-time RT-PCR

In order to confirm that AHS viral RNA was indeed extracted from the ECE yolk and cell culture supernatants, a RT-PCR was performed on the extracted RNA samples.

5.2.1.1 Primers

The primers described in Chapter 4 (*AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev*) were used for the amplification reactions.

5.2.1.2 RT-PCR

The SensiMix™ Two-Step Kit (Quantace: QT305-01) was used in all reactions according to the manufacturer's instructions, albeit scaled down. Volumes that follow are for one reaction. For the reverse-transcription reaction 2 µL of the extracted ECE RNA samples or 7 µL of the extracted cell culture RNA suspension was combined with 2 µL of a 50 µM solution of random hexamers, 1 µL of 10 mM dNTP mix and made up to 10 µL with DEPC-treated water if necessary, all on ice. The ECE samples were incubated at 95°C for 5 minutes and immediately chilled on ice for 2 minutes. The cell culture samples were incubated at 65°C for ten minutes followed by immediately freezing the mixture in liquid nitrogen for one minute and thawing on ice (Batten *et al.*, 2000). The 10 µL containing the RNA template was added to a separate tube containing 4 µL of the 5 × reverse transcriptase buffer, 1 µL RNase inhibitor (10 $u.\mu L^{-1}$), 0.25 µL reverse transcriptase buffer and 4.75 µL of DEPC-treated water to achieve a

total of 20 µL. The solution was incubated at 37-42°C for 50 minutes followed by 70°C for 15 minutes and immediately chilled on ice. All thermal cycling for the reverse-transcriptase (RT) reactions took place in an Applied Biosystems GeneAmp® 2700.

The amplification reaction was conducted as follows (for one reaction): 1 µL of the cDNA template from the RT reaction was combined with 7 µL sterile distilled water, 10 µL of SensiMix™ dT, 0.4 µL 50x SYBR® Green I solution, 0.8 µL each of 5 µM *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev* to achieve 20 µL. followed by a brief centrifugation. The reactions were carried out in a Corbett Rotor-Gene™ 6000 under the following conditions: Hold at 95°C for 10 minutes and 60 cycles of 95°C for 15 seconds, 50°C for 30 seconds, 72°C for 15 seconds. The ECE samples proceeded with a standard melt from 72-95°C. The cell culture samples were subjected to a High Resolution Melt immediately afterwards from 72-95°C at 0.1°C rise per step and the results analysed using the in-house Corbett Rotor-Gene™ 6000 Series Software 1.7©.

To confirm that the targeted 180 bp region was being amplified, a 1.2 % agarose gel containing 1 µg/µL ethidium bromide in 0.5 × TBE was loaded with the amplified samples (10 µL of sample was combined with 2 µL of loading buffer) and was run at 120V for 1 hour. The Fermentas O'GeneRuler™ 100 bp Plus, ready-to-use molecular weight marker (MWM) was used.

5.2.2 Amplicon DNA Sequencing

Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa) sequenced the resulting amplicons of the cell culture samples. The amplicons were first cloned into pJET1.2/blunt cloning vector and sequenced using standard primers. The returned sequences were edited to remove vector sequence and were correctly orientated and subjected to alignment analysis with *ClustalX2* and dendrogram analysis with *TreeView*.

5.3 Results

To confirm that the RNA extracted from the ECE yolks was AHS viral RNA, an RT-PCR was performed on the extractions using the primers selected in Chapter 4. The real-time and melt analysis results are seen in Figure 5.2. The real-time nature of the Corbett Rotor-Gene™ 6000 enables one to monitor the accumulation of product (White & Potts, 2006) (Figure 5.1). A single, diffuse band was evident with a leading edge of 182 bp (Figure 5.2).

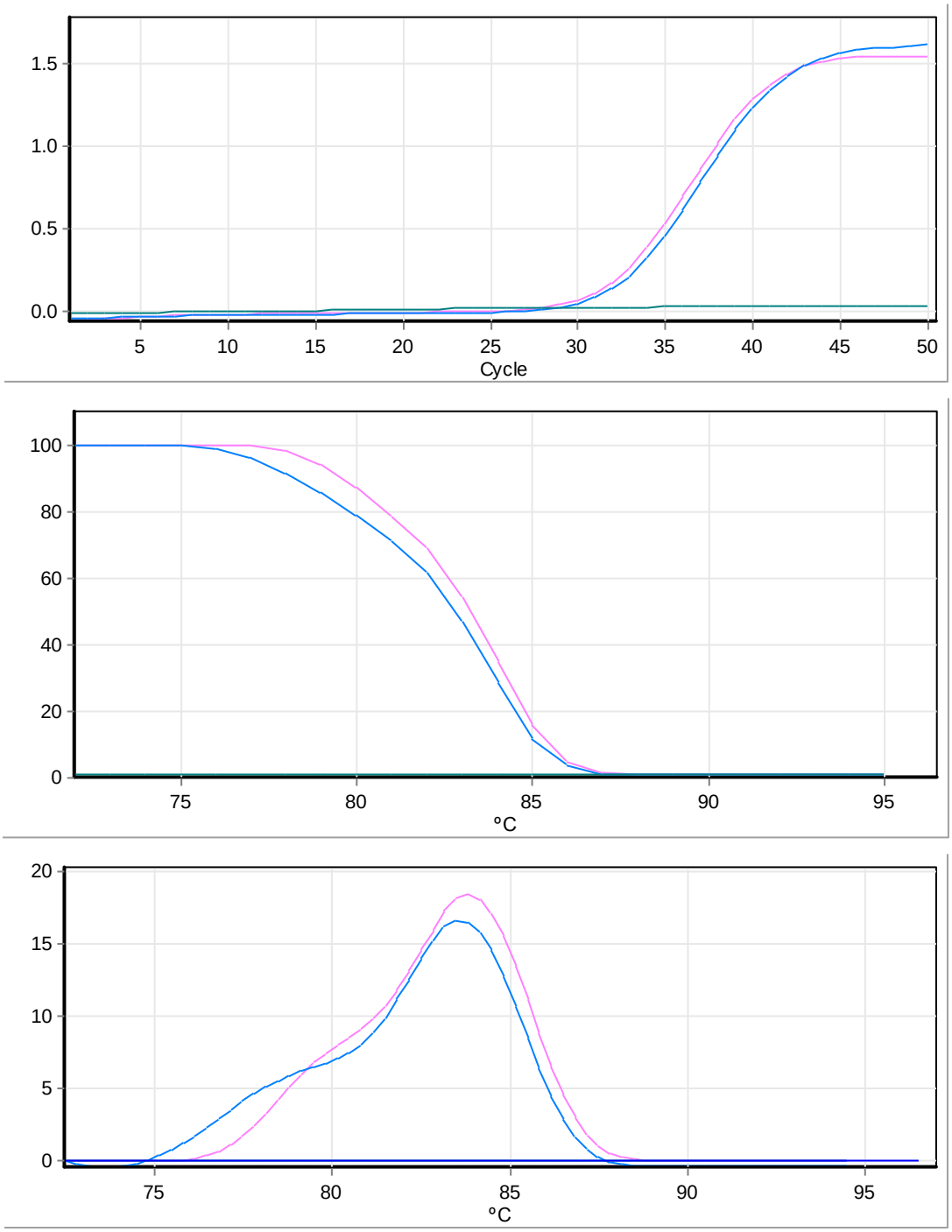


Figure 5.1: Real-time RT-PCR and HRM results for the 10-190 bp region of Segment 10 using the primer pair *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev*. Top: Fluorescence versus cycle number to determine amplification status of amplicon. Middle: Melt curve showing fluorescence versus temperature (°C). Bottom: Melt curve derivative versus the temperature. Amplicon amplified using the primers *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev*.
■ - TRIzol® extraction 1 (with no NaCl). ■ - TRIzol® extraction 2 (with NaCl). ■ - no template control.

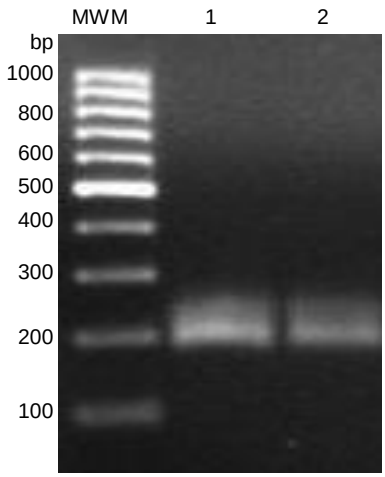


Figure 5.2: PCR products of TRIzol® extracted AHS viral RNA using the primers *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev*. 2 µL sample + 8 µL formaldehyde-containing loading buffer on an ethidium bromide containing 1.2 % (w/v) agarose gel. MWM: Fermentas GeneRuler™ 100 bp DNA Ladder. Lane 1: TRIzol® extraction 1 (with no NaCl). Lane 2: TRIzol® extraction 2 (with NaCl).

Melt peaks were calculated using the Rotor-Gene™ 6000 Series Software 1.7©. The melt curves seen in Figure 5.1 (bottom) peak at 83.8°C (■ – TRIzol® extraction 1 (with no NaCl)) and 83.5°C (■ – TRIzol® extraction 2 (with NaCl)). Product sizes in the gel and the corresponding peak melt temperature indicate that no improvement in the quality of the RNA extraction is achieved by the addition of NaCl to the isopropyl alcohol.

Figure 5.3 is the graph of the raw fluorescence data from the PCR of the AHS viral RNA extractions from the cell cultures and the primers *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev* that are designed to amplify a 180 bp product from genome Segment 10.

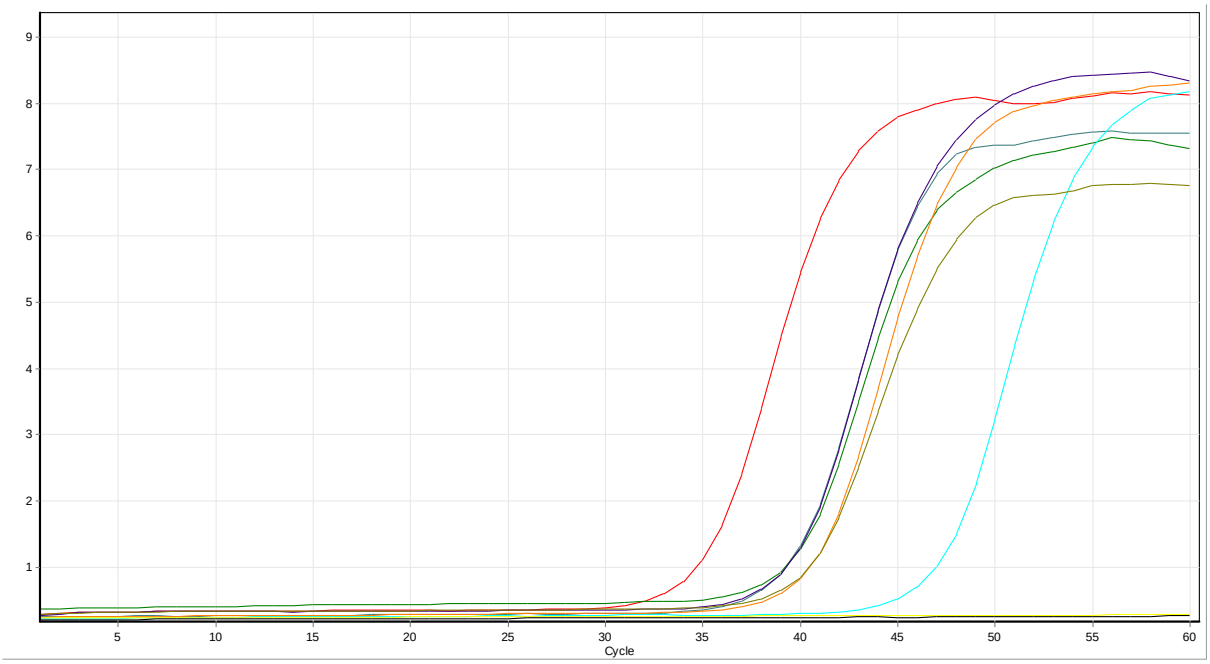


Figure 5.3: Corbett Rotor-Gene™ 6000 software raw data fluorescence curves for the amplification of the 10-190 bp region of AHSV Segment 10 using primers *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev*.
Where ■ - 1; ■ - 4; ■ - 5; ■ - 6; ■ - 7; ■ - 8; ■ - 9; ■ - Control; ■ - NTC.

Figure 5.3 clearly shows the amplification of product from cycle 30 until cycle 43, while Serotype 2 and 3 did not amplify. Amplified products were confirmed as being 180 bp by running a 1.2% (w/v) agarose gel containing 1 µg/µL ethidium bromide in 0.5 × TBE.

Ultimately, real-time PCR negates the need to run PCR products on gels. However, it was necessary in this instance in order to confirm that the correct product was being amplified. The primers *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev* have not been used before and it was necessary to confirm that they are amplifying a product of the correct size. All of the bands were sized and found to be 182 bp, which compares favourably with the expected size of 180 bp. The control from the cell culture did not result in a product, nor did the NTC.

The raw data from each data acquisition step is normalised by the Corbett Rotor-Gene™ 6000 software (Figure 5.4). The Control and NTC are unable to be suitably normalised.

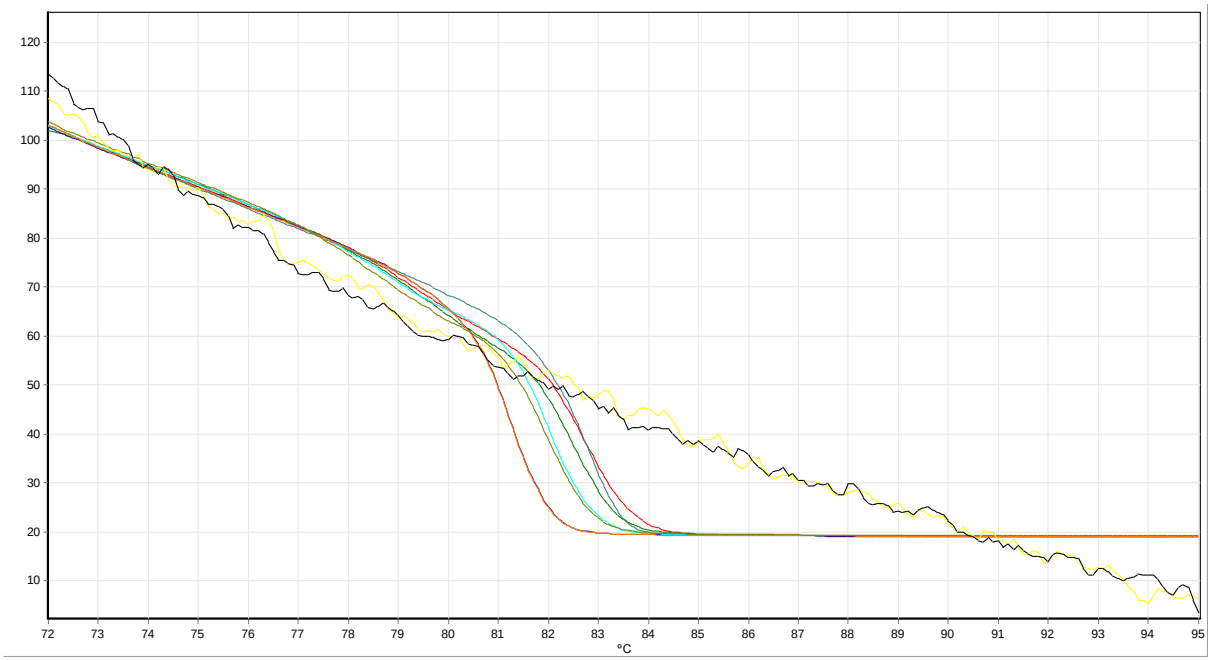


Figure 5.4: Normalised HRM curves of the 180 bp product of the targeted region of AHS viral genome Segment 10 for each of the serotypes, control and NTC.
Where ■ - 1; ■ - 4; ■ - 5; ■ - 6; ■ - 7; ■ - 8; ■ - 9; ■ - Control; ■ - NTC.

The derivative of the normalised graph measures the point at which the dissociation of the two DNA strands is most rapid – the peak melting temperature (Figure 5.5). A number of melt peak similarities/groupings can be distinguished: serotype 7 (■) and 8 (■); serotypes 6 (■) and 9 (■) and serotypes 1 (■) and 4 (■).

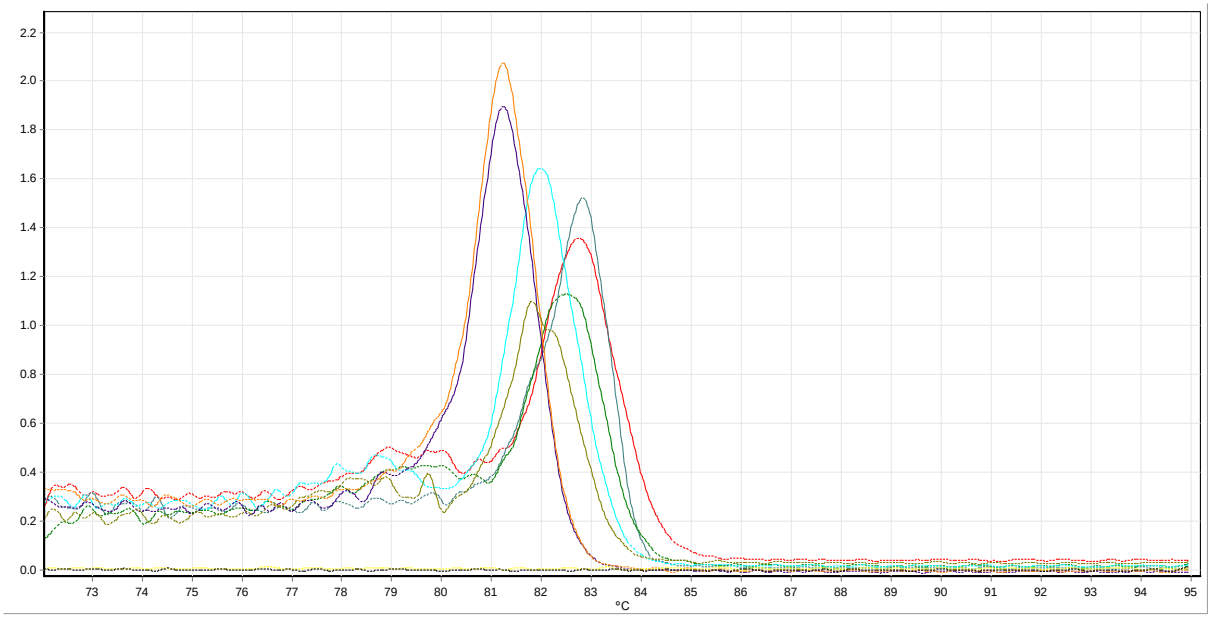


Figure 5.5: Corbett Rotor-Gene™ 6000 software HRM curves of the 180 bp product of the targeted region of AHS viral genome Segment 10 for each of the serotypes, control and NTC.
Where ■ - 1; ■ - 4; ■ - 5; ■ - 6; ■ - 7; ■ - 8; ■ - 9; ■ - Control; ■ - NTC.

Using the Corbett Rotor-Gene™ 6000 software, 'bins' can be defined for a set of melt curves. Melt peaks are clearly clustered into two, if not three distinct domains (Figure 5.6-5.8). In Figures 5.6-5.8, these have been defined as A (Figure 5.6; serotypes 7 and 8); B (Figure 5.7; serotypes 6 and 9) and C (Figure 5.8; serotypes 1, 4 and 5). Table 5.1 shows the results of the software's capabilities to call samples and place them in corresponding bins. In the developed assay, the bins would represent the nine serotypes.

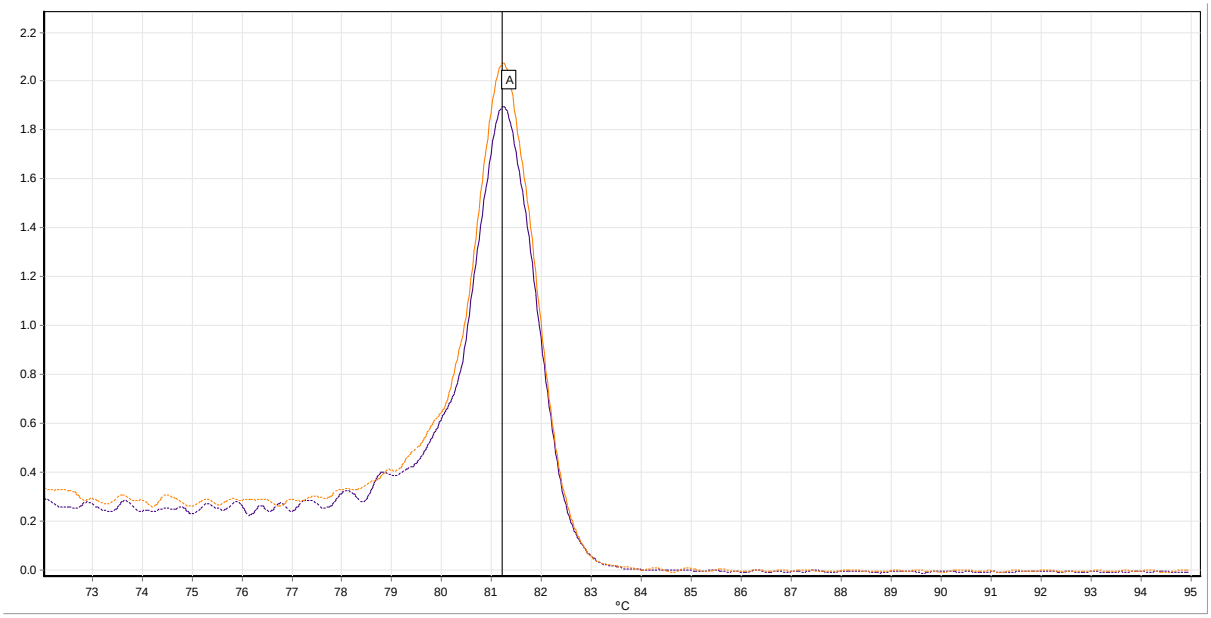


Figure 5.6: Corbett Rotor-Gene™ 6000 software HRM curves of serotype 7 and 8 AHSV Segment 10 10-190 bp – Group A
Where: ■ - 7; ■ - 8.

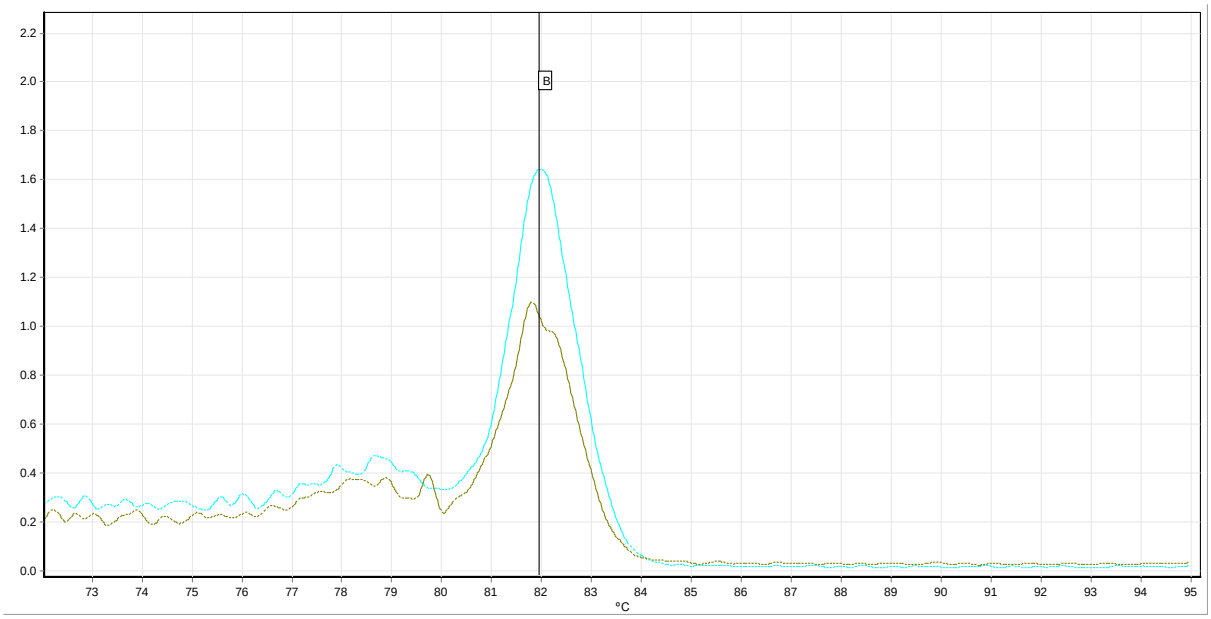


Figure 5.7: Corbett Rotor-Gene™ 6000 software HRM curves of serotype 6 and 9 AHSV Segment 10 10-190 bp – Group B.
Where ■ - 6; ■ - 9.

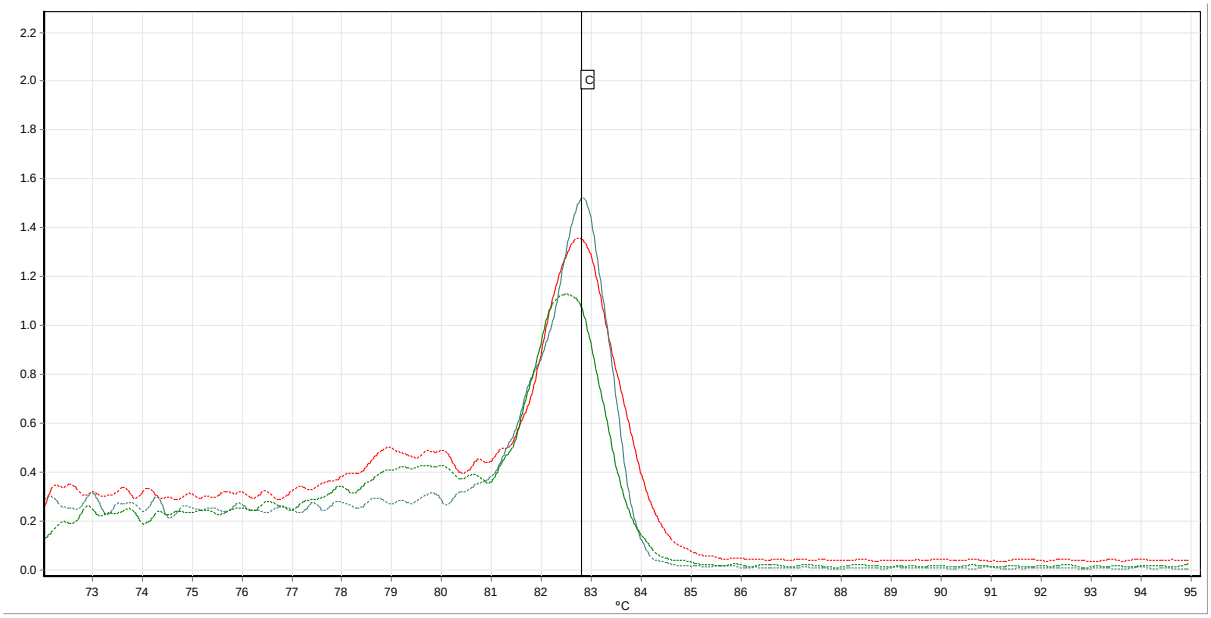


Figure 5.8: Corbett Rotor-Gene™ 6000 software HRM curves of serotype 1, 4 and 5 of AHSV Segment 10 10-190 bp – Group C.
Where ■ - 1; ■ - 4; ■ - 5.

Table 5.1: 10-190 bp amplified AHSV Segment 10 regions of each serotype and their automatically assigned bin using Corbett Rotor-Gene™ in-house software for the segregation of melt curves

Colour	Serotype	Maximum Peak (°C)	Bin
	1	82.75	(C)
	4	82.82	(C)
	5	82.48	(C)
	6	81.98	(B)
	7	81.23	(A)
	8	81.23	(A)
	9	81.80	(B)

The presence of 180 bp products on an agarose gel cannot be regarded as unequivocal proof of the successful amplification of the specified target region. The PCR products need to be sequenced in order to confirm with confidence that the correct target was amplified.

The resulting sequences (Figure 5.9) were edited to remove the vector sequences. In addition, due to some slight aberrations in the cloning, some of the sequences were

presumably truncated. Serotypes 7 and 9 contained small deletions in the 3′ terminal regions while half of serotype 5 appeared to have been deleted from the 5′ terminus and was therefore discarded. The sequences were therefore adjusted to take into account the deletions of serotypes 7 and 9. Identification of the nine serotypes was confirmed in this way.

```
AHSV6_Inqaba      ATCCCTTGTCATGAGTCTAGCTAGCATCTCCCAAAGCTATATGTCACATAATGAGAATGA
AHSV9_Inqaba      ATCCCTTGTCATGAGTCTAGCTAGCATCTCCCAAAGCTATATGTCACATAATGAGAATGA
AHSV4_Inqaba      ATCCCTTGTCATGAGTCTTGCTAGCATCTCCCAAAGCTATATGTCACATAATGAGAATGA
AHSV1_Inqaba      ATCCCTTGTCATGAATCTTGCTAGCATCTCCCAAAGCTACATGTCACATAATGAGAATGA
AHSV7_Inqaba      ATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAATGAAACTCA
AHSV8_Inqaba      ATCCCTTGTCATGAGTCTTGCTACGATCGCCGAAAATTATATGATGCATAATGGAAATCA
                  ************** *** ****  *** ** ***  ** ***   *******  * * *

AHSV6_Inqaba      AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV9_Inqaba      AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV4_Inqaba      AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV1_Inqaba      AATACCAATTGTACCATACGTTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV7_Inqaba      GAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCCGACGCTTGG
AHSV8_Inqaba      CAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCCGACGCTTGG
                   * * ********** **  **** ** ** ***    ** * *  ****** ******

AHSV6_Inqaba      TGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGAT
AHSV9_Inqaba      TGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGAT
AHSV4_Inqaba      TGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGAT
AHSV1_Inqaba      TGCATCCGCCAGTCAAATGGAGACCATGTCACTTGGGAT
AHSV7_Inqaba      TGGTCAGGCGGGTGGAATGGAGCCCATGTCGCTTGGGAT
AHSV8_Inqaba      TGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGAT
                  **     **  **  ******* ******* ********
```

Figure 5.9: Alignment of the AHSV Segment 10 10-190 bp amplicons chosen for sequencing showing the 3 base pair deletion in serotypes 6, 9, 4 and 1.

Following *ClustalX2* sequence alignment, dendrogram analysis with *TreeView* produced distinct groups (Figure 5.10) for serotypes 7 and 8, serotypes 6 and 9 and serotypes 1 and 4, which, although not in a distinct group are more closely related to each other than to any of the other serotypes. This is indicative of highly similar sequences within each group, distinct from the other groups. These features will also define the melt curves after HRM analysis.

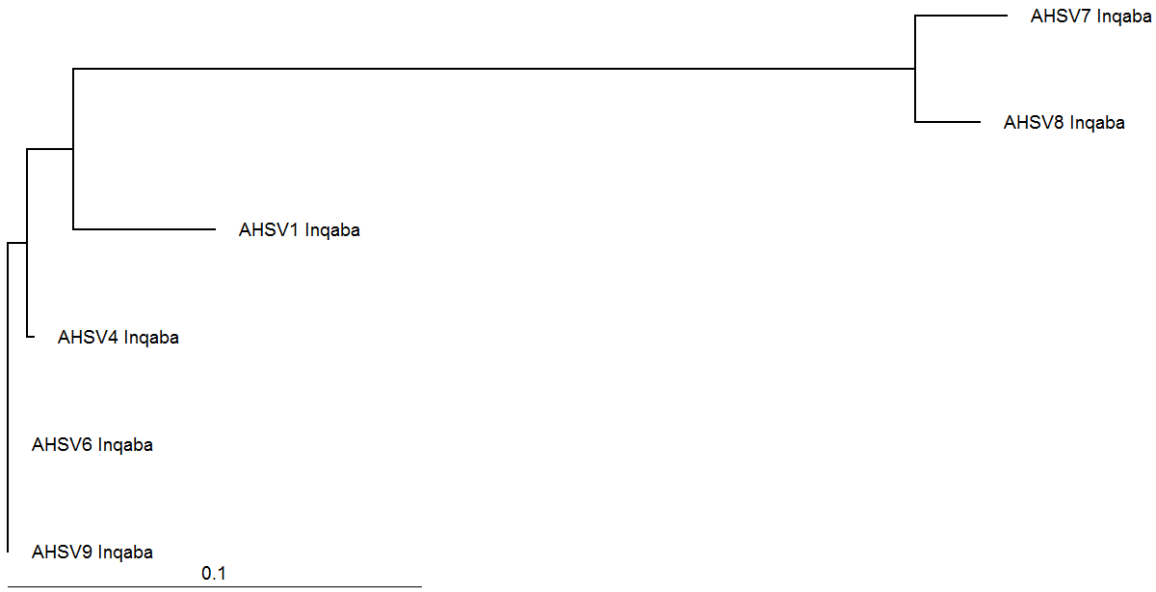
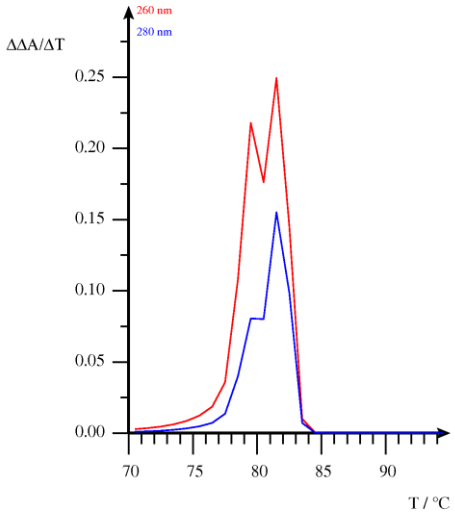
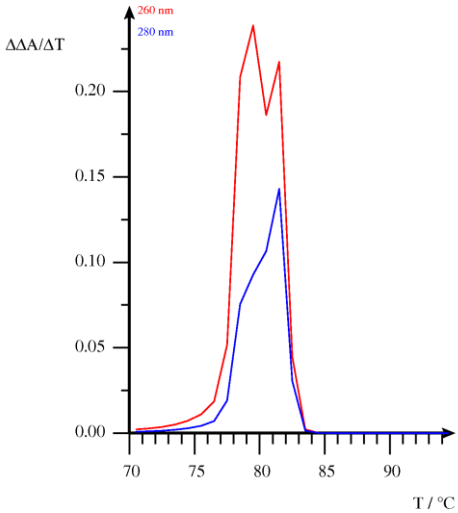


Figure 5.10: Dendrogram of the modified sequenced 180 bp AHSV genome Segment 10 target. The bar represents 0.1 substitutions per site.

To validate the use of POLAND for the predication of melt profiles, the 180 bp Segment 10 amplicons that were sequenced (Inqaba Biotechnical Industries (Pty) Ltd.,Pretoria, South Africa) were also run through POLAND, retrospectively (Figure 5.11). *In vitro* and *in silico,* it is clear that serotypes 1, 4, 6 and 9 have a similar melt profile (double peak), while serotypes 7 and 8 are similar (single peak). The approximate melt peaks are given in Table 5.2.



AHSV1_Inqaba



AHSV4_Inqaba

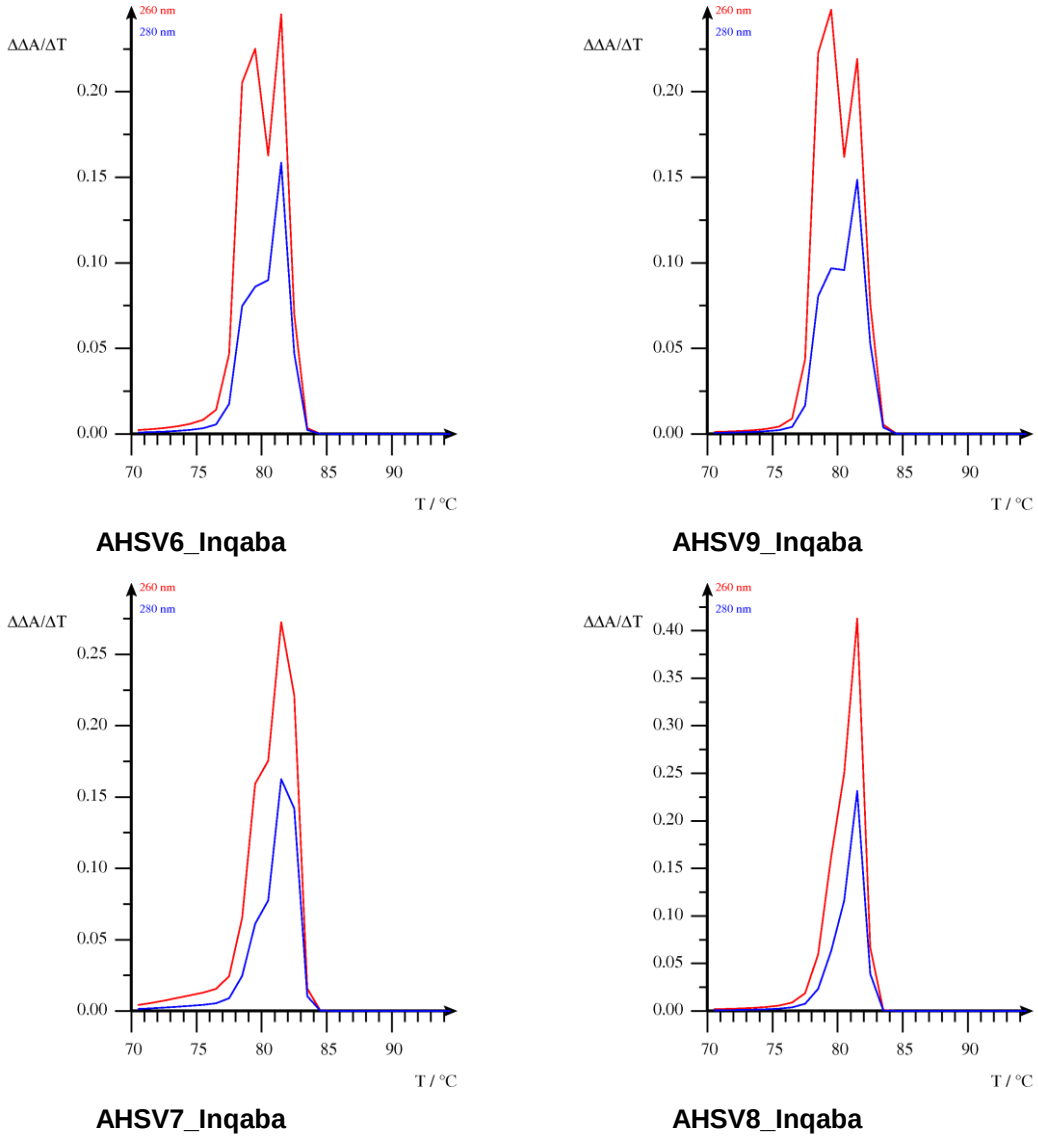


Figure 5.11: Predicted melt profiles of the sequenced (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa) 10-190 bp region amplicons according to POLAND. The serotype of each amplicon is found under each graph. The red and blue lines use the predicted absorbance readings at 260 nm and 280 nm respectively, to draw the graph and represent the change in absorbance over temperature.

Table 5.2: Melt peaks of the sequenced 10-190 bp amplicon of Segment 10 for the serotypes 1, 4, 6, 7, 8 and 9 according to POLAND

Serotype	Melt Peak (°C)
1	79 and 81
4	79.5 and 81.5
6	79.5 and 81.5
7	81.5
8	81.5
9	79.5 and 81.5

Although the melt profiles and peaks do not compare to the actual melt profiles and peaks from the Corbett Rotor-Gene™ 6000 software, the groupings are clearly common.

5.4 Discussion

Primers selected in Chapter 4 (i.e. *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev*) were used in RT-PCR on the ECE samples. A broad product was evident on the gel, with a leading edge of 182 bp (Figure 5.2). This compares favourably with the expected size of 180 bp. Examination of the sequences contained in AHS vaccine vial 1 (i.e. Serotypes 1, 3 and 4) reveals that serotype 1 contains 3 bases less than serotypes 3 and 4 (Figure 5.12). This would account for the variation on the agarose gels.

```
AHSV3_AJ007304        TGGAAATCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV3_D12479          TGGAAATCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV3_AJ007303        TGGAAATCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV4_U02712          TGGAGAGTCGGGGGCGATTGTCCCTTATGTGCCACCGCCATATAATTTCGCGAGCGCTCC
AHSV4_AJ007305        TGGAGAGTCGGGGGCGATTGTCCCTTATGTGCCACCGCCATATAATTTCGCGAGCGCTCC
AHSV4_Z48734          TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATGTTGCGAGTGCTCC
AHSV4_Z48735          TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSVref_AM883173      TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
AHSV1_U02711          TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
```

Figure 5.12: Sequences of serotypes 1, 3 and 4 (contained in AHS vaccine vial 1) showing the three base pair difference in serotype 1. `AHSVref` refers to serotype 1. The sequences displayed start with base number 61.

These experiments are the first in a series of investigations into the development of a rapid, cost-effective assay for the diagnosis and serotyping of the AHS virus. The rapidity and cost effectiveness of the assay will largely depend on the reagents used

and their respective protocols. For this reason, the ideal primer pair was chosen which would have the ability to amplify all nine serotypes, but produce a variety of melt profiles that would ideally be unique to each serotype. Initially, the chosen primer pair is only likely to result in a few common melt profiles. If these groupings in the melt profile matched the groupings of a dendrogram of the identical sequences, the proof of concept for the proposed assay under development would become a reality. Moreover, it would allow researchers to exhaust primer selection strategies *in situ*, and be confident that the primers selected would result in melt profiles that match the dendrogram groups. POLAND, as an *in silico* melt simulator, can be used to verify that dendrogram groupings from *TreeView* coincide with melt groupings *in vitro.*

A 2-step RT-PCR protocol was followed such that a stock of cDNA could be kept. The Quantace SensiMix™ 2-step kit had not been used before for an orbivirus, but the reagents are all standard and the reaction could be scaled down. Confirmation of the correct size amplicon was obtained by 180-182 bp bands visualised on a 1.2% (w/v) agarose gel. PCR products were sequenced by Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). The control and NTC did not produce any product.

A comparison of the 'bins' for AHS serotypes produced by HRMA on the Corbett Rotor-Gene™ 6000 in Figures 5.6-5.8, to the dendrogram produced by *TreeView*® (Figure 5.10) demonstrates that the serotypes correspond to the dendrogram groups (Figure 5.13). There is a definite relationship between the melt curves and the dendrogram groupings.

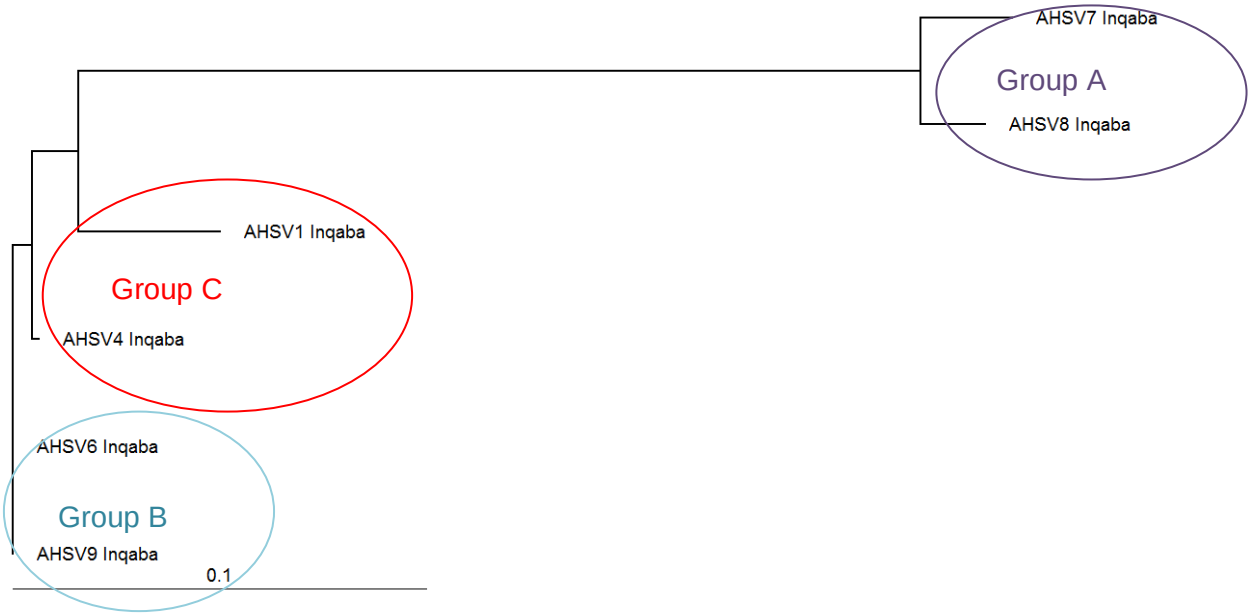


Figure 5.13: The three distinct groups of the sequenced AHSV Segment 10 amplicons correspond with the serogroups of the HRM melt profiles from the Corbett RotorGene™ 6000 for six of the nine analysed AHSV genome Segment 10 sequences. The bar represents 0.1 substitutions per site.

However, when the sequenced amplicons are aligned with the *GenBank* sequences of the AHSV Segment 10 10-190 bp regions and the dendrogram drawn, the serotypes do not correspond (Figure 5.14). The named serotypes of the reference strains do not appear to coincide with the serotypes of the *GenBank* sequences. However, the groupings (as in Figure 5.13) importantly, remain unchanged.

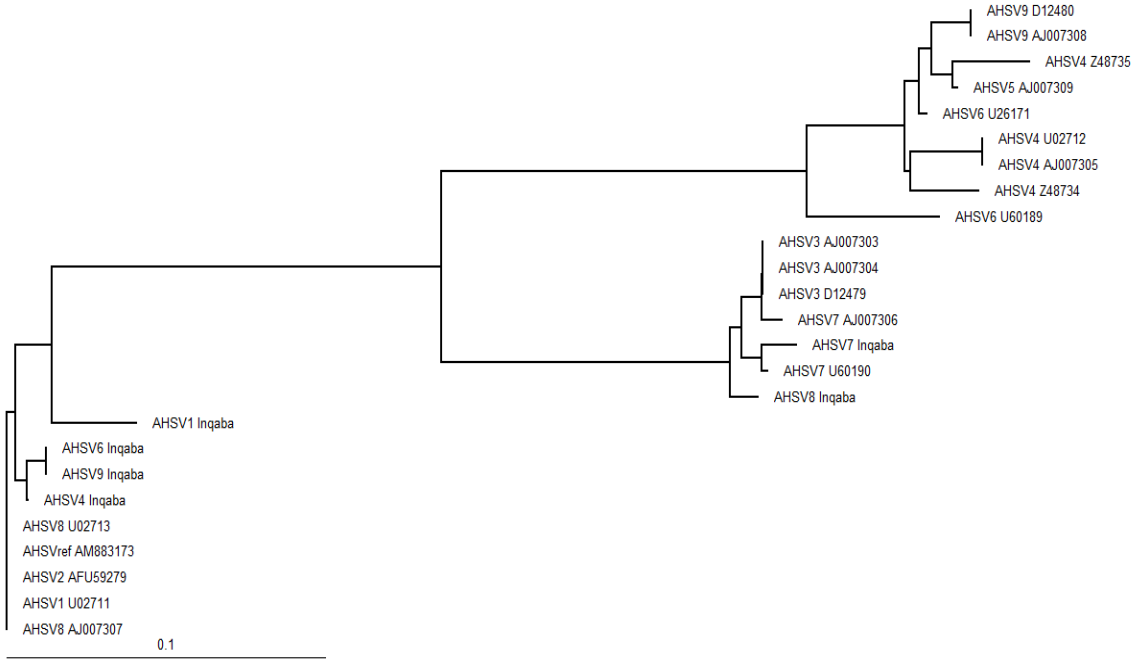


Figure 5.14: Dendrogram of the sequenced amplicons of the AHSV reference strains (obtained from the NICD) and the *GenBank* sequences for the identical region. The bar represents 0.1 substitutions per site.

Group A is situated with *GenBank* serotypes 3 and 7, group B is situated nearer *GenBank* serotypes 1, 2 and 8, while group C has been split, possibly due to the interference of the additional sequences. One possible reason may be viral evolution, although this is highly unlikely due to the genetically stable nature of the virus (Howell, 1962).

Interestingly, one of the features of the 10-190 region of AHSV genome Segment 10 is the presence of a three base pair deletion at position 94-96 of the amplicon (Figure 5.15):

```
AHSV7_Inqaba          GAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCCGACGCTTGG
AHSV7_U60190          GAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCCGACGCTTGG
AHSV3_AJ007303        GAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCCGACGCTTGG
AHSV3_AJ007304        GAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCCGACGCTTGG
AHSV3_D12479          GAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCCGACGCTTGG
AHSV7_AJ007306        GAGAGCAATTGTGCCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCCGACGCTTGG
AHSV8_Inqaba          CAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCCGACGCTTGG
AHSV6_Inqaba          AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV9_Inqaba          AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV4_Inqaba          AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV1_U02711          AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV8_AJ007307        AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV2_AFU59279        AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSVref_AM883173      AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV8_U02713          AAGATCAATTGTACCATACATTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV1_Inqaba          AATACCAATTGTACCATACGTTCCGCCACCGTAT---CATCCGACGGCTCCGGCGCTTGC
AHSV9_D12480          GGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCCGACGTTTTC
AHSV9_AJ007308        GGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCCGACGTTTTC
AHSV4_Z48735          GGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCCGACGTTTTC
AHSV5_AJ007309        GGGGACGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCCGACGTTTTC
AHSV6_U26171          GGAGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCCGACGTTTTC
AHSV4_U02712          GGGGGCGATTGTCCCTTATGTGCCACCGCCATATAATTTCGCGAGCGCTCCGACGTTTTC
AHSV4_AJ007305        GGGGGCGATTGTCCCTTATGTGCCACCGCCATATAATTTCGCGAGCGCTCCGACGTTTTC
AHSV4_Z48734          GGGGGCGATCGTCCCTTATGTGCCACCACCATACAATGTTGCGAGTGCTCCGACGTTTTC
AHSV6_U60189          AGAGGCGATCGTCCCATATGTGCCGCCACCATACAATTTCGCAAGTGCTCCGGCGCTTCC
                           * ** ** ** **  * ** ** ** **       * *  ****** ** **
```

Figure 5.15: 61-120 bp region of the 10-190 bp amplicon of AHSV Segment 10 to illustrate the three base pair deletion common to serotypes 1, 2 and 8 of the *GenBank* sequences and serotypes 4, 6 and 9 of the sequenced amplicons.

According to the sequences obtained from *GenBank*, this deletion belongs to serotypes 1, 2 and 8. The reference sequences from the NICD, however, show the identical deletion in serotypes 4, 6 and 9. Obtaining recent isolates from the Onderstepoort Veterinary Institute of all nine serotypes and repeating the same procedure may go some way to solving the anomaly. The additional, serotyped, strains of the virus will be included in the alignment and dendrogram (Figures 5.9 and 5.10 respectively) to determine which serotypes may contain the three base pair deletion to determine the source of the anomaly.

5.5 Conclusion

The primers that were selected after an intensive bioinformatic analysis (Chapter 4) successfully amplified the correct amplicons. They can thus be deemed suitable for HRM analysis. The primers *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev* were able to amplify the correct product of the AHS viral genome Segment 10 according to the gel analysis and sequencing results.

Serogroups were successfully defined in both the dendrogram analysis based on actual sequencing of the amplicons and in the High Resolution Melt curve analysis of the amplicon. Comparing the serogroups obtained from each method it is clear that there is a definite relationship between the dendrogram serogroups and the HRM serogroups and the two can be directly compared and equated. This thus constitutes the proof of concept for the further development of the proposed rapid, cost-effective AHS serotyping assay.

Chapter 6: CONCLUSION

An accurate and rapid diagnosis of African horse sickness is an important goal for researchers and veterinarians alike. Early detection of the virus and identification of serotype has immediate benefits that include applying the correct treatment regime for the animal, notifying authorities in the case of notifiable diseases and implementing suitable control measures to prevent further spread of the disease. The development of rapid assay methods to identify AHS serotypes is imperative in the study of the disease in order that epidemiological control might augment the more conventional prophylactic strategies currently employed. The ability to serotype an AHS outbreak as rapidly as possible, combined with the increasing development of monovalent vaccines, makes a rapid serotyping assay even more important (Koekemoer et al., 2000). As a result, the national equine population can be protected far more effectively against AHS. Furthermore, the serotyping and classification of the virus will assist greatly in a rapid classification of future outbreaks for taxonomic and epidemiological purposes.

The sero-prevalence of the virus and the vaccination coverage in the South African equid population are unknown (Lord *et al.*, 1997a) and remain so to this day. These problems are compounded by the lack of research funding for AHS. Expensive tests are required to understand the disease thoroughly and are therefore not performed, with many owners unwilling to pay for tests on a dead horse carrying already exorbitant veterinary expenses or rural horses that die without ever being reported. Tests to determine the serotype are undertaken only at Onderstepoort Veterinary Institute and take up to two weeks to achieve a result costing in excess of R1000. All of these factors compound the minimal number of epidemiological studies being undertaken which lead to often grossly misunderstood aspects of the disease. The assay under development will go some way to easing the financial and time constraints of studying an outbreak in real time. This has the potential to solve many of the unknowns surrounding AHS, particularly and most importantly, the role that each serotype plays in outbreaks and the form of the disease contracted by horses. In addition, the strain is not usually classified until sometime after the initial outbreak, as no system to rapidly classify the virus involved in the outbreak exists. In addition, it will benefit overall investigations and our understanding of the clinical disease (Abdalla *et al.*, 2002). The last two decades have seen a shift in the pursuit for rapid diagnostics from classical

microbiology to molecular biology based techniques, such as nucleic acid detection, particularly the polymerase chain reaction (PCR). The applicability of PCR is increased dramatically with the advent of real-time or quantitative PCR (Pusterla *et al.*, 2006). The system that is being developed is based on DNA amplification and High Resolution Melt (HRM) analysis that will detect the AHS virus and serotype it in a single test in a few hours and could potentially cost under R50 per sample.

However, no work exists that can be built on to develop this assay. Developing the protocol therefore requires essential preliminary investigations that constitute the bulk of this research. RT-PCR has previously been used to serotype AHSV (although not on clinical samples), but involved primer pairs for each serotype and would be impractical for routine field diagnosis (Sailleau *et al.*, 2000). As the first investigation, suitable primer pairs were selected after a rigorous bioinformatic analysis of the available AHSV Segment 10 sequences available on *GenBank*. Using *Clustal*X2 and *Primaclade*, the primers *AHS Seg10 10-190Fwd* and *AHS Seg10 10-190Rev* were selected. These primers were designed with a view to amplify a 180 bp product across all nine serotypes such that the amplicon of each serotype (or serogroup) was divergent. Therefore, the divergent regions would theoretically result in unique melt profiles. The choice of Segment 10 relied on the fact that it was divergent among the serotypes (with Segment 2). Segment 2, although responsible for serotype specificity for the virus, was too divergent and no region could be found that matched the criteria for the development of this assay.

It is unfortunate to note that due to a lack understanding and support amongst AHS researchers in South Africa, it proved almost impossible to obtain reference strains or recent isolates of the AHS virus from other workers in AHS in South Africa. Embryonated chicken eggs (ECE) represent an ideal environment to grow a virus without the need for highly specialised cell culture equipment. ECE were therefore inoculated with vaccine virus based on the protocol of Boorman *et al.* (1975). Despite the ease of the inoculation and growing of the virus, viral RNA proved very difficult to extract in a pure form from the egg yolk. No literature could be found pertaining to the extraction of viral RNA from egg yolk. Vero cells are preferred for the propagation of virus and extraction of viral RNA.

These strains were subjected to a RT-PCR using the primers designed in Chapter 3. Seven of the nine serotypes were successfully amplified. Serotypes 2 and 3 did not amplify. The reason is not yet known. A 180 bp product was achieved per serotype for the remaining serotypes. The RT-PCR was repeated with the same results, but requires exhaustive duplication to ensure the integrity of the primers and protocol. The PCR products were subsequently cloned and sequenced to confirm the correct target was amplified. The amplified sequences were subjected to a bioinformatic analysis using *Clustal*X2 to align them and *TreeView* to establish the sequence relationships in a dendrogram. Three distinct groups become evident.

The primary focus of the proposed assay under development is the use of High Resolution Melt (HRM) analysis to serotype the virus. The PCR products were subjected to HRM immediately subsequent to the PCR. The in-house software of the Corbett Rotor-Gene™ 6000 produces the melt curves. Of the seven serotypes that were amplified and melted, three groups of highly similar melt peaks evolved. The comparison of the three groups of melt peaks correspond very closely with the three groups of the phylogenetic analysis on the sequenced amplicons. This represents proof of concept for the assay under development.

However, this work represents only the preliminary investigations of this assay. Through the funding of UKZN Innovation (Pty) Ltd. and the provisional patent filed through the UKZN Intellectual Property and Patents Office, the assay can now be developed to its fullest potential such that a rapid, cost-effective serotyping assay for AHS becomes available to the South African endemic market and the international non-endemic market.

6.1 Future Work

The primers selected as part of these preliminary investigations can be improved upon for serotype differentiation. The single primer pair used in this dissertation has enabled proof of concept to be established. As part of the ongoing process, additional primers and multiplexing will be investigated. Different approaches to primer design will separate the individual serotypes from their groupings. Future strategies may include the use of two primer pairs that divide the nine serotypes into two groups. Asymmetrical PCR and probes have a very real potential to segregate serotypes.

Although Segment 10 was chosen for these preliminary investigations, Segment 2 cannot be excluded from primer design strategies in the future. RT-PCR and the HRM protocols will be optimised in the context of the assay. The real-time aspect of the instrumentation used will also allow a quantitative assay to be developed in conjunction with a serotyping assay. This will jointly provide researchers and veterinarian alike with a level of viraemia that may be related to the serotype of the infecting virus.

Recent isolates of the nine serotypes were recently acquired and these will undergo the cell culture and RT-PCR protocols as for the reference strains. These serotyped isolates may provide an explanation for the anomaly between the sequenced amplicons and the *GenBank* sequences where the serotypes appear incorrectly placed. It is also envisaged that the vaccine virus can be subjected to the same treatments such that vaccinated and field strain infected equines may be differentiated.

In keeping with the mandate for a rapid and cost-effective assay, the extraction of viral RNA from the blood of infected equines will be pursued. Investigations into various other extraction procedures will be conducted and additional methods to purify the virus from *in vitro* and field samples will be examined. Examples include the Whatman FTA® cards and magnetic nanoparticles (Chen *et al.*, 2006).

6.2 Prospects

The work contained in this dissertation comprises preliminary investigations into the development of a novel assay to rapidly diagnose and serotype African horse sickness using molecular biology techniques. This work has been identified as intellectual property and a provisional patent (Application No. 2009/04542) has been filed through the Intellectual Property and Technology Transfer Office of the Research Office of the University of KwaZulu-Natal (June 2009). Proof of concept, that molecular diagnostics can differentiate between AHSV serotypes is provided by this work and provides a foundation for optimisation and validation of the assay. A business model such as that entered for the UKZN Institutional Phase of the National Innovation Competition 2009 (2nd place) will see the exciting prospects of this rapid diagnostic assay for influencing prophylaxis, early warning systems, and epidemiological modelling and therefore control of African horse sickness.

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Appendix 1

CLUSTAL alignment of full-length AHSV Segment 2

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AHSV8_DQ868775      GTTTAATTCATCATGGCGTTCGAGTTTGGCATATTACTAACTGAGAAAGTAGAAGGTGAT
AHSV8_AY163333      GTTTAATTCATCATGGCGTCGGAGTTTGGCATATTACTAACTGAGAAAGTAGAAGGTGAT
AHSV5_AY163331      GTTTATTTCATCATGGCTTCAGAGTTTGGCGTTCTGTTGACCGATAAAGTTGAAGGCGAT
AHSV6_DQ868774      GTTAAATTCACCATGGCTTCCGAATTTGGCATTTTGATTTGTGATAAATTAAAGGAAAAC
AHSV6_NC_005996     GTTAAATTCACCATGGCTTCCGAATTTGGCATTTTGATTTGTGATAAATTGAAGGAAAAT
AHSV9_DQ868776      GTTTAATTCACCATGGCGTTCGGAATACTTCAGACGGACAAAATTAGAGAGAAT
AHSV4_EU046574      GTTTAATTCACCATGGCGCCCGAGTTTGGAATATTGATGACAAATGAAAAATTTGACCCA
AHSV4_DQ868773      GTTTAATTCACCATGGCGTCCGAGTTTGGAATATTGATGACAAATGAAAAATTTGACCCA
AHSV4_D26570        GTTTAATTCACCATGGCGTCCGAGTTTGGAATATTGTTGACAGATGAAAAATTTGACCCG
AHSV1_FJ011108      GTTTATTTCAGCATGGCGTCTGAATTTGGAATTCTATTGACCGAGAGAATCTTTGACGAA
AHSV1_AY163329      GTTTATTTCAGCATGGCGTCTGAATTTGGAATTCTATTGACCGAGAGAATCTTTGACGAA
AHSV2_AY163332      GTTTATTTCAGCATGGCGTCTGAATTTGGAATACTTTTCACCGAAAAGATCTATGACCAA
AHSV1_Z26316        GTTTAATTCACCATGGCTTCGGAATTCGGGATCCTATTGACAAATCAAATATATGATCAA
AHSV3_U01832        GTTTAATTCACCATGGCTTCGGAATTCGGGATCCTATTGACAAATCAAATATATGATCAA
AHSV3_DQ868772      GTTTAATTCACCATGGCTTCGGAATTCGGGATCTTATTGACAATTCAAATATATGATCAA
AHSV7_AY163330      GTTTAATTCACTATGGCTTCTGAGTTTGGAATTTTGTACACTGATCAGATCTACGAACAA
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AHSV8_DQ868775      GCACTGGAAAAGACGAATTGTGAAGTGATAATAACGAAAAACGGGAGAGTGAAACATAAA
AHSV8_AY163333      GCACTGGAAAAGACGAATTGTGAAGTGATAATAACGAAAAACGGGAGAGTGAAACATAAA
AHSV5_AY163331      GCTTTAGAGAAAACGAATTGTGAAGTAATTCTTACACGAAGTGGTCGCGTACGGCGGAGG
AHSV6_DQ868774      ACCTTGGAAAAAACGAATTGTGACGTTATTATTACGGGAGTAGGAAAGGTGAGTGTACGC
AHSV6_NC_005996     ACTTTAGAAAAAACGAATTGTGACGTTATTATTACGGGAGTGGGAAAAGTAGGTGTACAC
AHSV9_DQ868776      ACACTCGAAAAAACAAATTGTGATGTGATTCTTACGAAAGAAAATAGAGTGCGGATGAAA
AHSV4_EU046574      AGCATAGAGAAAACCATTTGCGATGTTATAGTTACGAAGAAGGGAAGAGTGAAGCATAAA
AHSV4_DQ868773      AGCTTAGAGAAAACCATTTGCGATGTTATAGTTACGAAGAAGGGAAGAGTGAAGCATAAA
AHSV4_D26570        AGTTTAGAGAAGACCATTTGCGATGTTATAGTTACGAAGAAGGGGAGAGTGAAACATAAA
AHSV1_FJ011108      ACATTGGAAAAAACAAATTGTGATGTTATTATAACCGAGGAGAAGAAAGTAAAACGGAAG
AHSV1_AY163329      ACATTGGGAAAAACAAATTGTGATGTTATTATAACCGAGGAGAAGAAAGTAAAACGGAAG
AHSV2_AY163332      ACGTTGGAGAAAACGAATTGTGATGTGATTATCACTGAGGAGAGGAAAGTGAATCGGAGA
AHSV1_Z26316        ACATATGAGAAAGAGATGTGTGATGTAATTATTACAGCGGAGAATGCAGTTAGAAGAGTT
AHSV3_U01832        ACATATGAGAAAGAGATGTGTGATGTAATTATTACAGCGGAGAATGCAGTTAGAAGAGTT
AHSV3_DQ868772      ACATATGAGAAAGAGAAGTGCGATGTAATTATTACAGCGGAAAATGCAGTTAGAAGAGTT
AHSV7_AY163330      ACATTGGAGAAGACGAGCTGTGACGTGATCGTAACAAAAGAGAATGCTGTAAAGAGGGTC
                          *  **    *  ** ** ** **  * **           **

AHSV8_DQ868775      GAGGTTGATGGAGTAAAAGGCTATGAGTGGGAATTTACAGACCATAGGCTGGGGCTCTGC
AHSV8_AY163333      GAGGTTGATGGAGTAAAAGGCTATGAGTGGGAGTTTACAGACCATAGGCTGGGGCTCTGC
AHSV5_AY163331      GAGGTTGACGGAGTTAAAGGATATGAATGGGAATTTACAGATCATCGATTAGGATTATGT
AHSV6_DQ868774      GAAGAAGACGGCATATTAGGTTACGAGTGGGAAGAGACTAATCATAGATTGGGATTGTGC
AHSV6_NC_005996     GAAGAGGACGGTGTGTTAGGATATGAGTGGGAGGAGACTAACTATAGGTTAGGATTGTGC
AHSV9_DQ868776      GAGGTCGAAGGAGTGAAAGGATATTATTGGGAGGACACCGATCACAGGTTAGGTTTATGT
AHSV4_EU046574      GAGGTGGATGGCGTATGTGGATACGAGTGGGATGAAACGAATCACCGATTCGGATTGTGT
AHSV4_DQ868773      GAGGTGGATGGCGTATGTGGATACGAGTGGGATGAAACGAATCACCGATTCGGATTGTGT
AHSV4_D26570        GAGGTGGATGGCGTGTGTGGATACGAGTGGGATGAAACGAACCACCGATTTGGATTGTGT
AHSV1_FJ011108      GAGGTCGAGGGAGTGTTGGGTTACGTGTGGGAGGAAACTAACCATAGGTTCGGCTTATGC
AHSV1_AY163329      GAGGTCGAGGGAGTGTTGGGTTACGTGTGGGAGGAAACTAACCATAGGTTCGGCTTATGC
AHSV2_AY163332      GAGGTGGAAGGAGTGCGGGGATATGTATGGGAAGAAACAAACCACCGTTTTGGATTATGT
AHSV1_Z26316        GAGGTTGCGGGAGTACATGGTTATGAGTGGGGTGCGACGAATCATAGGCTTGGGTTGTGT
AHSV3_U01832        GAGGTTGCGGGAGTACATGGTTATGAGTGGGGTGCGACGAATCATAGGCTTGGGTTGTGT
AHSV3_DQ868772      GAGGTTGCGGGAGTATATGGTTATGAGTGGGGTGCGACGAATCATAGGCTCGGGTTGTGT
AHSV7_AY163330      GAAATCGATGGCGTGCTAGGGTATGAGTGGGGCGCAACAAATCACCGATTAGGACTATGC
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AHSV6_DQ868774      GAAATAGAAAATACGGTATCAATCAGTGATTTCGTCTATAAGCAAATAAGATGTGAAGGA
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AHSV9_DQ868776      GAGGTCGAACATACTGTGTCTGTGAGGGATTTTGTGTATAAACAAACGAAATGTGAAGGA
AHSV4_EU046574      GAGGTGGAACACGACATGTCTATATCGGAATTTATGTACAATGAGATCAGATGTGAGGGG
AHSV4_DQ868773      GAGGTGGAACACGACATGTCTATATCGGAATTTATGTACAATGAGATCAGATGTGAGGGG
AHSV4_D26570        GAGGTGGAACACGATATGTCAATATCGGAGTTTATGTACAATGAGATCAGATGTGAGGGG
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AHSV1_AY163329      GAAGGAAATTATGATTTGGCATTTTCAGATACGATGTATTGCCAAACAAAGTGCGACGGA
AHSV2_AY163332      GAGAATTCATTTGACGAAAAAATATCGGAAACCATGTATTGCCAAATTAAGTGTGAAGGT
AHSV1_Z26316        GAGGTAGAGAACACGAAGTCGATTGGGAGAATGATTTATGAACAGATTCGATGCGAAGGT
AHSV3_U01832        GAGGTAGAGAACACGAAGTCGATTGGGAGAATGATTTATGAACAGATTCGATGCGAAGGT
AHSV3_DQ868772      GAGATAGAGAATACGAAGTCGATCGGGAGAATGATTTATGAACAGATTCGATGCGAAGGT
AHSV7_AY163330      GAAATAGAACACGTAAAGACGATATCTGAGTTCATGTATGAACAAATTAAATGTGAGGGT
                    **                    *          * **    * *    ** ** **

AHSV8_DQ868775      GCGTATCCGGTGTTTCCGCATTATATTACTGATGTTTTGAAGTATGGTGTCATGGTTGAT
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AHSV3_DQ868772      GCGTACCCAATTTTTCCGCACTATATAACAGATACTTTAAAATATGGGAAATCAATTGAT
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AHSV8_AY163333      AGGAATGATCACCAAATAAGAGTGGATAGGGACGTGAAAGAATTAGGAAAGATTTTGATC
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AHSV6_DQ868774      AGAAACGATCATCAGATCCGAGTTGACAGAGATGAAAAAAGTATTGGAAAAATCCAGATT
AHSV6_NC_005996     AGAAATGATCATCAGATCCGAGTTGACAGAGATGAAAAAAGTATTGGAAAAATCCAGATT
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AHSV4_D26570        AGGAATGATCATCAGATTAGAGTGGACAGAGACGATAATGAAATGAGGAAAATATTGATA
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AHSV3_DQ868772      AGAAATGACAACCAAATAAGGGTGGATAGGGACGACGAACGGCTCCGCAAAATAAAAATC
AHSV7_AY163330      AGAAATGATAATCAAGTAAGAGTAGATAGAGATGATGAAAGACTACAAAAAATAAGATC
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AHSV8_DQ868775      CAACCGTACTTTGGAGAGGTGTTCTTTTCACCTGAGTTTTATACATCGACCTTTTTGAAA
AHSV8_AY163333      CAACCGTACTTTGGAGAGGTGTTCTTTTCACCTGAGTTTTATACATCGACCTTTTTGAAA
AHSV5_AY163331      CAACCATACTTCGGTGAGGCGTACTTTTCACCAGAATTTTACACCTCAACTTTTTTGAAG
AHSV6_DQ868774      CAGCCGTATTTCGGCGATATGTATTTCTCCCCCGAGTATTACCCGGCAACTTTCGTTAAA
AHSV6_NC_005996     CAGCCGTATTTCGGCGATATGTATTTCTCCCCCGAGTATTACCCGGCAACTTTCTATTAAA
AHSV9_DQ868776      CAGCCGTATTTAGGCGACGCTTATTTTTCGCCAGAGTATTACACTGCTACCTTCTTTAAA
AHSV4_EU046574      CAGCCGTATGCAGGTGAGATGTACTTTTCGCCGGAATGTTATCCGAGCGTTTTTCTTCGG
AHSV4_DQ868773      CAGCCGTATGCAGGTGAGATGTACTTTTCGCCGGAATGTTATCCGAGCGTTTTTCTTCGG
AHSV4_D26570        CAGCCGTATGCGGGTGAGATGTATTTTTCGCCAGAAATGTTATCCGAGCGTTTTCCTTCGG
AHSV1_FJ011108      CAGCCGTATTTGGGTGAAATGTATTTTTCGCCGGAGAACTATTCAACTGTATTTTGCAAA
AHSV1_AY163329      CAGCCGTATTTGGGTGAAATGTATTTTTCGCCGGAGAACTATTCAACTGTATTTTGCAAA
AHSV2_AY163332      CAGCCGTATATGGGTGAAATGTATTTCTCACCCGAGAGCTATTCTACCGTTTTCTGTAAG
AHSV1_Z26316        CAGCCGTATTTTGGAGAAATGTACTTTTCACCAGAAAATTATATAACAGTTTTTTGTAAA
AHSV3_U01832        CAGCCGTATTTTGGAGAAATGTACTTTTCACCAGAAAATTATATAACAGTTTTTTGTAAA
AHSV3_DQ868772      CAGCCGTATTTTGGAGAAATGTACTTCTCACCAGAAAATTATATAACAGTTTTTTGTAAA
AHSV7_AY163330      CAACCATATTTTGGAGAAGCGTTCTTCTCGCCGGAGACCTACTCTTCAACTTTTTGCAAA
                    ** ** **    ** **    *  ** ** ** **    **          **

AHSV8_DQ868775      AGACAAGCGATTAATAGTGATGTTGAGATGTTGAGGAGATCGATTCCGAAACGGATAAAA
AHSV8_AY163333      AGACAAGCGATTAATAGTGATGTTGAGATGTTGAGGAGATCGATTCCGAAACGGATAAAA
AHSV5_AY163331      AGACAGGCGATTCAAATGAATGTTGAGATGCTGAGAGCGTTTGTACCAAAAAGGGTGGCT
AHSV6_DQ868774      CGTGAGCCTCTGCCAATCTCAGTAGATATGATCAGAGATTATATTGGAGCCAGAATGCGA
AHSV6_NC_005996     CGTGAGTCTTTACCAATCTCAGTAGATACGATCAGAGGTTATATTGGAGCTAGAATGCGA
AHSV9_DQ868776      AGAGAACCGCTGCCAATTCATGTGGATATGATTCGGGATTATATCGGAAAACGAAT-TAA
AHSV4_EU046574      AGGGAAGCGCGAAGTCAAAAGCTTGATCGGATTCGGAATTATATTGGAAAGAGAGTCGAA
AHSV4_DQ868773      AGGGAAGCGCGAAGTCAAAAGCTTGATCGGATTCGGAATTATATTGGAAAGAGAGTCGAA
AHSV4_D26570        AGGGAGGCGCGAAGCCAAAAGCTTGATCGAATTCGGAATTACATTGGAAAAAGAGTCGAA
AHSV1_FJ011108      AGGCAAGCGCTGGCACTTGGAGTTGACGATCTAAGACATTCTGTTGATGTAAGGAATGAG
AHSV1_AY163329      AGGCAAGCGCTGGCACTTGGAGTTGACGATCTAAGACATTCTGTTGATGTAAGGAATGAG
AHSV2_AY163332      AGACAAGCAGAGCTCATGTCAATTGAAGATCTAAGGTATCCATTTGATATAAGATGCGAC
AHSV1_Z26316        AGGCAGGCAATCAGTGGCCAAATTGAGGTTTCTCGTTCAATTATAGGTCGGCGGATGAAG
AHSV3_U01832        AGGCAGGCAATCAGTGGCCAAATTGAGGTTTCTCGTTCAATTATAGGTCGGCGGATGAAG
AHSV3_DQ868772      AGGCAGGCAATCAGCGGCCAAATTGAGGTTTCTCGTTCAATTATAGGCCGGCGAATGAAG
AHSV7_AY163330      AGACAAGCGATTAGAGGGCACGTTGCGAAGATGCGAACGTTTGCAAAGGATAGGATTGAT
                     *  *  *             * *         *               *

AHSV8_DQ868775      TACTTTGAAGACCAGATGGAGTTAAGGAAAAGCGTAAACGGAAATTGGATCGGTACGTTA
AHSV8_AY163333      TACTTTGAAGATCAGATGGAGTTAAGGAAAAGCGTAAATGGAAATTGGATCGGTACGTTA
AHSV5_AY163331      TTTTATGAAGACGATATGAGGAGGGGCGGTACTATTAACGGGAATTGGATTGGAGCGTTA
AHSV6_DQ868774      AAGATTGAAGCCAGGGCGGGACGAATTAAGGAGGGTGGTGGAAATTTGTTGGAGTGCGCG
AHSV6_NC_005996     GGGATTGAAGCTAGGGCTGGGCGAATTAGGGAGGGTGATGGAAACTTGTTAGAATGCGCG
AHSV9_DQ868776      TTATTTTGAGCGAGAATTAAGTGG--TGGGGTGCGAGATGCAAATTTAGAGATGATAGTT
AHSV4_EU046574      TTTTATGAAGAGGAGAGTAAGAGAAAAGCAATCCTTGATCAGAATAAGATGTCTAAGGTT
AHSV4_DQ868773      TTTTATGAAGAGGAGAGTAAGAGAAAAGCAATCCTTGATCAGAATAAGATGTCTAAGGTT
AHSV4_D26570        TTTTTTGAAGAGGAGAGTGGGAGAAAGGCGATCCTTAATCAGAATAAGATGTCTAAGATT
AHSV1_FJ011108      TTCGAAGAAACGAATCACCAAACGAAAGGTGTGTTGAATGGAAATAAACAGAGAGCGCTT
AHSV1_AY163329      TTCGAAGAAACGAATCACCAAACGAAAGGTGTGTTGAATGGAAATAAACAGAGAGCGCTT
AHSV2_AY163332      TTCGAAGAAACTTCGTTCCAAACGAAATCCTCTTTAGATGGTAAGAAGCTTAGATTATTG
AHSV1_Z26316        TATGAAGAGAGTGCTGAACAGACGAAGGGAACGATAAACGCAAATAAATATCGATTGTTG
AHSV3_U01832        TATGAAGAGAGTGCTGAACAGACGAAGGGAACGATAAACGCAAATAAATATCGATTGTTG
AHSV3_DQ868772      TATGAAGAGAGCGCTGAACAGACGAAGGGAACGATAAACGCAAATAAATATCGATTGTTG
AHSV7_AY163330      TTTGAAGAGAGTAGCGCACAGACGAAAT--ACG-TCAATGGAAATAAGGTTAAGATTTTA
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AHSV8_DQ868775      CACAAATGGAAAGAGAGCGTAGATGCGCGTATGTTA-GAGGAGGGAGTGGGAAAGAAAGT
AHSV8_AY163333      CACAAATGGAAAGAGAGCGTAGATGCGCGTATGTTA-GAGGAGGGAGTGGGAAAGAGAGT
AHSV5_AY163331      CAGGCGTGGAAGGAAAAAGCGGATTTGCAAATGTCGCGCGAGGGGAATAGTCAAACTAAT
AHSV6_DQ868774      CGCAGATGGGAAAAAGCGGCATATGAGAGAATTGAG-AATGAAAGAGCAT--TACGGTGT
AHSV6_NC_005996     CGCAGATGGGAAAAAGCGGCGTATGAGAGAATTGAG-AATGAAAAGCGT--TGCGGTGC
AHSV9_DQ868776      GAGAAGTGGAAAGATAATACTTATAAAAGGATTGAA-GGTGAGAAAACGA--CGATGTGC
AHSV4_EU046574      GAACAATGGAGAGATGCGGTTAATGAAAGGATTGTG-AGTATCGAACCAAAGCGAGGTGA
AHSV4_DQ868773      GAACAATGGAGAGATGCGGTTAATGAAAGGATTGTG-AGTATCGAACCAAAGCGAGGTGA
AHSV4_D26570        GAGCAGTGGAGAGATGCGGTTAATGAAAGGATTGTA-AGTGCCGAACCAAAGCGAGGTCA
AHSV1_FJ011108      GAAGTTTGGAAGGAAATGGCGTATCAACGGATGCGTAAGGAGGGATCGCGGGGTAGATGT
AHSV1_AY163329      GAAGTTTGGAAGGAAATGGCGCATCAACGGATGCGTAAGGAGGGATCGCGGGGTAGATGT
AHSV2_AY163332      GAAAAATGGAAACGGGCTTCGCAAGAACGCATGCACGAAGAAAACGACCGTGGAAAGTGT
AHSV1_Z26316        GAGAAGTGGCGCGATCTGGCCTATGAACAAATTGAAATAGAACGTGATAATGAAAGATGT
AHSV3_U01832        GAGAAGTGGCGCGATCTGGCCTATGAACAAATTGAAATAGAACGTGATAATGAAAGATGT
AHSV3_DQ868772      GAGAAGTGGCGTGATCTGGCTTATGAGCAAATTGAAATGGAAGGTAGTAGTGAAAGGTGT
AHSV7_AY163330      GAGGAGTGGAAGGGGCGGACTGATGCTAGAATGTTGATGGAGGGCCAACAAGAGAAGTGC
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AHSV8_DQ868775      GTGTGTAAGCCACGAAACTGATGTGGTATATCAGCTAATGAAAAAGATGCGGTTTGGTCT
AHSV8_AY163333      GTGTGTAAGCCACGAAACTGACGTGGTATATCAGCTAATGAAAGAGATGCGGTTTGGTCT
AHSV5_AY163331      -TGCGTTGACCATAATGCGGATGTAATATATCAACACATGAAGAAGTTAAGATTTGGATT
AHSV6_DQ868774      GTGGTG----CATGAAACAGATCCGACGTATCAAATTCTTAAAAAACTAAGATTTGGATT
AHSV6_NC_005996     GTGGCA----CATGAAACAGATCCAACGTATCAAATTCTTAAAAAACAAAGATTTGGATT
AHSV9_DQ868776      GTGAGA----CATGAACCAGACTCAGTGCTACAGATGTTGAAAAAGATGAGGTTTGGGAT
AHSV4_EU046574      GTGCTATGATCACGGAACCGACATTATCTACCAATTCATAAAAAAGCTGAGATTTGGAAT
AHSV4_DQ868773      GTGCTATGATCACGGAACCGACATTATCTACCAATTCATAAAAAAGCTGAGATTTGGAAT
AHSV4_D26570        ATGCTATGATCATGGTACCGACATTATCTACCAATTCATAAAAAAGCTAAGATTCGGAAT
AHSV1_FJ011108      ATCGGA----CACGATGATGATGTAATGTACCAACTGATAAAGAAATTGAGATATGGGAT
AHSV1_AY163329      ATCGGA----CACGATGATGATGTAATGTACCAACTGATAAAGAAATTGAGATATGGGAT
AHSV2_AY163332      GCTGGT----CACGACGAAGATGTGTTGTATCAATTAGTTAAGAAATTAAGGTATGGCCT
AHSV1_Z26316        TTGACG----CATAACACCGACCCGATTTATCAATTGATAAAAAAGATGAGGTATGGAAT
AHSV3_U01832        TTGACG----CATAACACCGACCCGATTTATCAATTGATAAAAAAGATGAGGTATGGAAT
AHSV3_DQ868772      TTGACG----CATAACACTGATCCGATTTATCAATTGATAAAGAAGATGAGGTTTGGAAT
AHSV7_AY163330      GTTGCA----CATGAGGTTGATCCGATTTACCAATTGATTAAAAAGATGAGATATGGAAT
                              **       **          **     * **  *     * *  **  *

AHSV8_DQ868775      ATTATACCCCCATTATTATATGCTAAACAATGAGTATGTAGTGAAGAAAGAGAATGTTGA
AHSV8_AY163333      ATTATACCCCCATTATTATATGCTAAACAATGAGTATGTAGTGAAGAAAGAGAATGTTGA
AHSV5_AY163331      ACTGTATCCGCATTACTATATGCTCAATCCAGAATATACGGTTGAAGAAAAGAGCAAAGG
AHSV6_DQ868774      CATTTATCCGCATTATTATGTCTTGAATACTGACTATAATCCAACAACTGTGACTAGAAC
AHSV6_NC_005996     TGTCTATCCGCATTATTATGTTTTGAATACTAATTATAATCCAACAACGGTGACTAGAAC
AHSV9_DQ868776      GTTATATCCAAACTACTATATGCTGAATACAGATTATATAGTTACTGAATCAAGTAAGGA
AHSV4_EU046574      GATGTACCCACACTATTATGTTTTGCATAGTGATTACTGTATTGTACCAAATAAGGGGGG
AHSV4_DQ868773      GATGTACCCACACTATTATGTTTTGCCTAGTGATTACTGTATTGTACCAAATAAGGGGGG
AHSV4_D26570        GATGTACCCACATTATTATGTTCTGCATAGTGACTATTGTATTGTACCAAACAAAGGCGG
AHSV1_FJ011108      GATGTATCCTCATCATTACGCATTGAATGCTCGATATGAGGTTTCAAACCCAAGCGC---
AHSV1_AY163329      GATGTATCCTCATCATTACGCATTGAATGCTCGATATGAGGTTTCAAACCCAAGCGC---
AHSV2_AY163332      ACTGTATCCACATAGCTATACCCTTAACACGAAATATAAAATTGTCAACCCAAGCGT---
AHSV1_Z26316        GATGTATCCAGTGCACTACATGCTAAACGATAGATATAAAGTTGTGCAAGAGCGCGCAGA
AHSV3_U01832        GATGTATCCAGTGCACTACATGCTAAACGATAGATATAAAGTTGTGCAAGAGCGCGCAGA
AHSV3_DQ868772      GATGTATCCAGTGCATTACATACTAAACGATAAATACAAGGTCGTACAAGAGCGCGCAGA
AHSV7_AY163330      GATGTATCCTACGCATTATATGTTGAGCCAGAGATATAGGATTATATCTGAACGTCGAAA
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AHSV8_DQ868775      CGCGTTAATTGGTAGTTGGTTGATCAAGGAGAGATCGAGTGGAAAGGCAGAGTACTCCCA
AHSV8_AY163333      CGCGTTAATTGGTAGTTGGTTGATCAAGGAGAGATCGAGTGGAAAGGCAGAGTACTCCCA
AHSV5_AY163331      AGGTTTGATAGCCAACTGGTTAGTGAAAGAGAAGGCGGCGGGACGTGCTGAGAATTCACC
AHSV6_DQ868774      TTCGCGGATTAATGATTGGTTGCTAAAAGAAAAGACACAAGGAGTTGTGAAGGCCGCTGA
AHSV6_NC_005996     TTCGCGGATTAATGATTGGTTGCTAAAAGAAAAGACACAAGGAGTTGTGAAGACCGCTGA
AHSV9_DQ868776      GGCGCCGTTAAATCGATGGTTAGTGAAGGAGAAAACAGTTGGGAAAGTTAAAGCGGCCGA
AHSV4_EU046574      AACTAGTATTGGATCATGGCATATAAGAAAACGTACTGAGGGTGATGCGAAAGCTTCTGC
AHSV4_DQ868773      AACTAGTATTGGATCATGGCATATAAGAAAACGTACTGAGGGTGATGCGAAAGTTTCTGC
AHSV4_D26570        CACAAGTATCGGATCATGGCATATAAGAAAACGTACTGAAGGTGACGCGAAAGCTTCCGC
AHSV1_FJ011108      GGCACGGATTAAAGATTGGCTGTT---GAAGGTAAGGGTTAATGTAGGGAGGGCACAGGA
AHSV1_AY163329      GGCACGGATTAAAGATTGGCTGTT---GAAGGTAAGGGTTAATGTAGGGAGGGCACAGGA
AHSV2_AY163332      TTCACAGATTAAGGACTGGCTGTT---AAAAACAAGGGATGGTATTAGAGAAGAATCTGG
AHSV1_Z26316        TATGGGGGTTGAAAAGTGGCTTCTTCAAAAAATCGGAAGGGGTACCCAAAGGCGGAAGGC
AHSV3_U01832        TATGGGGGTTGAAAAGTGGCTTCTTCAAAAAATCGGAAGGGGTACCCAAAGGCGGAAGGC
AHSV3_DQ868772      TATGGGGATTGAAAAGTGGCTTCTTCAAAAAATCGGAAGGGGTACCCAAAGGCGGAAGGC
AHSV7_AY163330      AATGGGAATTGAGAAGTGGCTATTGGAGAAAGTTGAAAGGGGAGTACAGAGAGGAGTAGC
                                *      ***    *     *

AHSV8_DQ868775      GATGTATTCAGGGGTGGGACCGCTTAGTGGGTTACGCGAACGTATCGAAAAGGATGAGTT
AHSV8_AY163333      GATGTATTCAGGGGTAGGACCGCTTAGTGGGTTACGCGAACGTATTGAAAAGGATGAGTT
AHSV5_AY163331      AATGTATTCGGGGGTCGGACCGCTAAATACATCGCGTGAGCGTATCGAGAGGGATGAGTT
AHSV6_DQ868774      GGCGTATTCCGATAACGCGGAGTTGAAAACTTTAGCGGAACGAATGGAGGAGGAGGAACT
AHSV6_NC_005996     GGCGTTTTCCGACAACGCAGAGTTGAAGACTTTAGCGGAACGAATGGAAGAGGAGGAGCT
AHSV9_DQ868776      AGCTTTTGCGGGTAATTCGTTATTAAAAAGCTTGGCGAGCCGGATGGAGGACGAGGAGTT
AHSV4_EU046574      TATGTATTCTGGAAAAGGTCCACTGAATGACTTACGAGTTAAAATTGAGCGGGATGATTT
AHSV4_DQ868773      TATGTATTCTGGAAAAGGTCCACTGAATGACTTACGAGTTAAAATTGAGCGGGATGATTT
AHSV4_D26570        CATGTATTCTGGAAAGGGTCCGCTGAATGACTTACGAGTTAAAATTGAGCGGGATGATTT
AHSV1_FJ011108      GAAAGCTGATCAAACAGGCCCGCTCGCCGAGATGGCTAGATCAATTGAAAACGATGAATT
AHSV1_AY163329      GAAAGCTGATCAAACAGGCCCGCTCGCCGAGATGGCTAGATCAATTGAAAACGATGAATT
AHSV2_AY163332      AATATCAGATAAGCAGGGACCATTAGCGGAGTTGGTGAGTGCAATCAAAGACGAGGAGCT
AHSV1_Z26316        TGACGATGGCGATAATGATGCGCTCTTACAGCTAGAAAGAATGATGAGCAGTGAAGAGCT
AHSV3_U01832        TGACGATGGCGATAATGATGCGCTCTTACAGCTAGAAAGAATGATGAGCAGTGAAGAGCT
AHSV3_DQ868772      TGACGATGGCGATAATGATACGCTCTTACAGCTAGAAAGAATGATGAGCAGTGAGGAGCT
AHSV7_AY163330      CGGTGCGAAAGGTAATGAATCGTTAGTACGGTTAGAAAAACTGATGCAGAAAGAAGAGCT
                                       *                    **       ** **  *

AHSV8_DQ868775      GGACGAAAAAGTTATTCAAGAGATTATCGCCTATGGCTCAAAATTTAGCACATATACAGG
AHSV8_AY163333      GGATGAAAAAGTTATTCAAGAGATTATTGCCTATGGCTCAAAATTTAGCACATATACAGG
AHSV5_AY163331      AGATGAAAAAGTTATTCAGGAAATCATTGCTTATGGATCCAAATTTAGCACCTACGCGGG
AHSV6_DQ868774      GACTGTGGATATCATTCGGGCGGTAATCAGGTATGGGGCCAAATATGCTACGCGCTCGGG
AHSV6_NC_005996     GACTGAGGATATTATTCGAGCAGTGATCAGGTATGGAGCCAAATACGCTACGCGCTCGGG
AHSV9_DQ868776      GAGTCGAGAAATAATAATTGCAGTGATCAATTATGGTTCGAAATTTGGAACTAGATCTGG
AHSV4_EU046574      ATCTCGAGAGACAATTATTCAGATCATTGAGTACGGTAAGAAATTTAATTCATCAGCAGG
AHSV4_DQ868773      ATCTCGAGAGACAATTATTCAGATCATTGAGTACGGTAAGAAATTTAATTCATCAGCAGG
AHSV4_D26570        ATCTCGAGAGACGATTATTCAGATTATCGAATACGGTAAAAAATTTAATTCATCTTCAGG
AHSV1_FJ011108      AAGCCGTCAAGTGGTTGACCAAATTATACAATATGGTGGACAATTTAGTTCATGCTCAGG
AHSV1_AY163329      AAGCCGTCAAGTGGTTGACCAAATTATACAATATGGTGGACAATTTAGTTCATGCTCAGG
AHSV2_AY163332      AAGTCGGGGAGTAATTGGGCGGATCGTACAATATGGCTCGCAATTTAGTTCGTGTGCAGG
AHSV1_Z26316        GGAAAGATCCGTTATTGAGAGCGTCATAAGATTCGGTTCTTTATATAACGCGCACGCGGG
AHSV3_U01832        GGAAAGATCCGTTATTGAGAGCGTCATAAGATTCGGTTCTTTATATAACGCGCACGCGGG
AHSV3_DQ868772      AGAAAGACCCGTTATTGAGAGCGTCATAAGATTCGGTTCCTTATATAACGCGCACGCGGG
AHSV7_AY163330      TGAGAATTCTGTGATCGAGAATGTAATACGCTACGGTTCGCAGTTTAGCACACACGCCGG
                                  *       *  *    *  **       *      *     * **
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AHSV8_DQ868775      TGCCAAGCAC-GGTGATATATCGCTAAAAGACTTAGTTGAGTATTGTGAAAGTTTAACCA
AHSV8_AY163333      TACCAAGCGC-GGTGATATATCGCTAAAAAACTTAGTTGAGTATTGTGAAAGTTTGACCA
AHSV5_AY163331      TAC-ACGCACAGGAGATCTCACTTTGAATGAATTAGTAAAGTATTGCGAAAGTTTAACGA
AHSV6_DQ868774      TATGCGAGAG-GATACACTGTCACTCCAGGAGTTGGATCGTTATTGCGATTCCCTAACAA
AHSV6_NC_005996     TATGCGAGAG-GATACACTGTCACTCCAGGAGTTGGATCGTTATTGCGATTCTCTAACAA
AHSV9_DQ868776      AAAGAAGAAG-GATTTGATGACTATTGATAAGCTTGAAAAATATTGTGATTCGTTGACGA
AHSV4_EU046574      TGATAAGCAG-GGGAACATTTCAATTGAAAAATTGGTAGAGTATTGTGATTTTTTGACAA
AHSV4_DQ868773      TGATAAGCAG-GGGAACATTTCAATTGAAAAATTGGTAGAGTATTTTGATTTTTTGACAA
AHSV4_D26570        TGATAAGCAG-GGGAATATTTCAGTTGATAAATTGGTGGAGTATTGTGACTTCTTGACAA
AHSV1_FJ011108      CGCACGTGAA-GATGATATTCCGATAAATATTTTGTTGGAGTATTGTGAGTCCTTAACAA
AHSV1_AY163329      CGCACGTGAA-GATGATATTCCGATAAATATTTTGTTGGAGTATTGTGAGTCCTTAACAA
AHSV2_AY163332      GGAACGAGAA-GATGATATCCCGATCGAAACATTGATTCGTTACTGCGACTCGCTGACGA
AHSV1_Z26316        CAAAAAGACA-GGCGACATTCCATTAGAAGTCTTGATTAAATATTGCGATTCGCTTACTA
AHSV3_U01832        CAAAAAGACA-GGCGACATTCCATTAGAAGTCTTGATTAAATATTGCGATTCGCTTACTA
AHSV3_DQ868772      CAAGAAGACA-GGCGACATTCCATTAGAAGTCTTGATAAAATATTGCGATTCGCTTACTA
AHSV7_AY163330      AGAAAAGACT-AATGATATGCCTCTGTCCGTTTTGATAAAATACTGCGAATCATTAACCA
                                                  *  *  *       *      ** *  **     * ** *

AHSV8_DQ868775      CATTCGTACACAAGAAGAAAAAAGAT---GGTGAGGAGGAAACCGCACGACAGTTTTTTA
AHSV8_AY163333      CATTCGTACACAAGAAGAAAAAAGAT---GGTGAGGAGGAAACCGCACGACAGTTTTTTA
AHSV5_AY163331      CTTTTGTTCATAAGAAAAAGAAGGAG---GGAGAGGATGAGACTGCAAGGGAGTTTTTCA
AHSV6_DQ868774      CTTTTGTTCATAAAAAGAAGAAGGATGAAGGTGATGATGAAACAGCACGTACGATAATTA
AHSV6_NC_005996     CTTTTGTTCATAAAAAGAAGAAGGATGAAGGTGATGATGAAACAGCACGTACGATAATTA
AHSV9_DQ868776      CGTTCGTCCACAAAAAGAAGCGTGACGAAGGTGACGATGAAACAGCTAGGGCTATCATTA
AHSV4_EU046574      CATTCGTTCATGCGAAGAAGAAAGAAGAGGGTGAGGATGATACTGCTCGACAGGAGATAA
AHSV4_DQ868773      CATTCGTTCATGCGAAGAAGAAAGAAGAGGGTGAGGATGATACTGCTCGACAGGAGATAA
AHSV4_D26570        CATTTGTCCACGCGAAGAAGAAAGAAGAGGGTGAAGATGATACCACTCGACAGGAGATAA
AHSV1_FJ011108      CATTTGTTCACAGAAAGAAGAGAAAGGAGGGGGATGATCTCACTGCACGCAATACCTTTC
AHSV1_AY163329      CATTTGTTCACAGAAAGAAGAGAAAGGAGGGGGATGATCTCACTGCACGCAATACCTTTC
AHSV2_AY163332      CATTTGTTCATCGAAAGAAGAGAGAACGAGGCGATGATACCACAGCGCGTGACGTCTTCA
AHSV1_Z26316        CATTCGTGCATAAAAAGAACAGAGAGGGAGGAGATAATCAGACCGCTCGCGACGAGATTA
AHSV3_U01832        CATTCGTGCATAAAAAGAACAGAGAGGGAGGAGATAATCAGACCGCTCGCGACGAGATTA
AHSV3_DQ868772      CATTCGTGCATAAAAAGAACAGAGAGGGAGGAGATAATCAGACCGCTCGCGACGAGATTA
AHSV7_AY163330      CTTTTGTACATAAAAAGAATCGCGAGGGAGGCGATAATCAGACGGCGCGTGATGAGATTA
                                                  * ** ** **    ** **     *    ** **  *    **  *  *       *

AHSV8_DQ868775      AAAACAAGTGGATACAGGGGATGCCCAAGATGAATTTTGAAAGTGAGATGAAGGTATCTC
AHSV8_AY163333      AAAACAAGTGGATACAGGGGATGCCCAAGATGAATTTTGAAAGTGAGATGAAGGTGTTTC
AHSV5_AY163331      AAAGTAAATGGATACAAGGTATGCCAAAGATGAACTTTGAAAATGAAATGATTATGTCAA
AHSV6_DQ868774      GAAATCAGTGGATTAAGGGAATGCCACGAATGGATTTCAAAAAAGAGATGAAAATCACAC
AHSV6_NC_005996     GAAATCAGTGGATTAAAGGGATGCCTCGAATGGATTTCAAAAAAGAGATGAAAATCACTC
AHSV9_DQ868776      GAAACCAATGGATTAAGGGGATGCCGAGTATGAATTTGAAAAAAGAAATGAAAGTTTCGC
AHSV4_EU046574      GAAAAGCATGGGTTAAGGGGATGCCTTATACGGATTTCTCAAAACCGATGAAAATCGCGC
AHSV4_DQ868773      GAAAAGCATGGGTTAAGGGGATGCCTTATATGGATTTCTCAAAACCGATGAAAATCACGC
AHSV4_D26570        GAAAAGCATGGGTTAAGAGGATGCCTTATATGGATTTCTCAAAACCGATGAAAATAACGC
AHSV1_FJ011108      GGCAGGCGCTGATTAAGAGTATGCCAACCATGAATTTCAAGAATCAAATGAAAATGAC--
AHSV1_AY163329      GGCAGGCGCTGATTAAGAGTATGCCAACCATGAATTTCAAGAATCAAATGAAAATGAC--
AHSV2_AY163332      GAAATGCCTTAGTTGGAAGTATGCCTAAAATGGACTTCAAGAATCAAATGAAGATGGCCA
AHSV1_Z26316        GGAGGGCAGTGGTAAAGAATATTCCATCGATGAAACAGGAGAATCAGATGAAAGTCACTC
AHSV3_U01832        GGAGGGCAGTGGTAAAGAATATTCCATCGATGAAACAGGAGAATCAGATGAAAGTCACTC
AHSV3_DQ868772      GGAGGGCAATGGTAAAGAATATTCCATCGATGAAGCAGGAAAATCAGATGAAAGTCACTC
AHSV7_AY163330      GAAAAGCTATGGTGCATAATATGCCGACCATGAATCACAACAACCCGATGAAGGTGACGA
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AHSV8_DQ868775      GCGGACCATGGGCTAACATTCAATTCTTCTGGAGCATTGATATGTTTAAAAGAAACAATG
AHSV8_AY163333      GCGGACCATGGGCTAACATTCAATTCTTCTGGAGCATTGATATGTTTAAAAGAAACAATG
AHSV5_AY163331      GAAAGTCATGGGCGAATACAAAATTCTTTTGGAGTATTGATATGTTCAAGAGAAATAATG
AHSV6_DQ868774      GAGGCCCGATTGCGAACTGGTCGTTTTTTATGTCTATAGATGCATTTAAAAGAAACAATA
AHSV6_NC_005996     GAGGTCCGATTGCGAACTGGTCGTTTTTTATGTCTATAGATGCTTTCAAAAGGAATAATA
AHSV9_DQ868776      GTGGTCCTATTCAAAATTGGTCGTTTTTCATGTCCCTAGAGATGTTTAAACGTAATAACA
AHSV4_EU046574      GTGGATTCAACAGAAATATGCTTTTCTTTGCGGCGCTCGATTCATTCAGAAAGAGGAACG
AHSV4_DQ868773      GTGGATTCAACAGAAATATGCTTTTCCTTGCGGCGCTCGATTCATTCAGAAAGAGGAACG
AHSV4_D26570        GTCGGTTCAACAAGAATATGCTTTTCTTTGCGGCACTCGATTCATTCAGAAAGAGGAACG
AHSV1_FJ011108      GAGAGGGTGGGGAAATTATAC-ATTTTTCTCATATATTGATAGATTCAGTCGTATTTATA
AHSV1_AY163329      GAGAGGGTGGGGAAATTATAC-ATTTTTCTCATATATTGATAGATTCAGTCGTATTTATA
AHSV2_AY163332      AAGAGGGTGGGGCAACTATACCGTTCTTTTCATACATTGATAGGTTTAGCCGGACTTACA
AHSV1_Z26316        CTAATATCAGG--AATTTT-CTGTTTTTCGCATATTTAAACGGTTTTAAAAGGAATAATG
AHSV3_U01832        CTAATATCAGG--AATTTT-CTGTTTTTCGCATATTTAAACGGTTTTAAAAGGAATAATG
AHSV3_DQ868772      CAAATATCAGG--AATTTT-CTGTTTTTCGCATATTTAAACGGTTTTAAAAGGAATAATG
AHSV7_AY163330      AGAACTTTAAG--AACTTT-CTCTTTTTCGCGTATTTAGATGGCTTTAAAAGAAATAACG
                                *       **  *       *  *    ** *     *   *

AHSV8_DQ868775      GAGTGGATATAGATCCCAACGGCGAAAACTGGAAAAAATATAAGGCGGAAGTCCAAGAAC
AHSV8_AY163333      GGGTGGATATAGATCCGAACGGCGAAAATTGGAAAAAATATAAGGCGGAAGTCCAAGAAC
AHSV5_AY163331      GCGTTGATATTGATCCAAACGGTAAGAATTGGAAAGATTACAAGAAAAAGGTACAAGAGC
AHSV6_DQ868774      AGGTCGACATTAATCCGAACCACCAAACGTGGAAGGATCATATCAAAGAAGTTACTGATC
AHSV6_NC_005996     AGGTTGATATTAATCCGAATCACCAGACGTGGAAGGATCACATTAAAGAAGTGACTGATC
AHSV9_DQ868776      AAGTTGATATTGATCCGAATCATGATACGTGGAAAAATCACGTTAAAGAGATCAGGGAGA
AHSV4_EU046574      GTGTAGATGTTGATCCGAATAAGGGTAAGTGGAAAGAACATATAAAGGAGGTAACCGAAA
AHSV4_DQ868773      GTGTAGATGTTGATCCGAATAAGGGTAAGTGGAAAGAACATATAAAGGAGGTAACCGAAA
AHSV4_D26570        GCGTAGACGTTGATCCAAATAAGAATAAGTGGAAAGAACATATAAAAGAGGTAACCGAAA
AHSV1_FJ011108      ATATGAATATTGATCCGAATGGTAAGCTTTGGATTGAACATAAACAAACGGTGAGTGAGC
AHSV1_AY163329      ATATGAATATTGATCCGAATGGTAAGCTTTGGATTGAACATAAACAAACGGTGAGTGAGC
AHSV2_AY163332      AGATGAATATTGACCCGAATAAGGGTTTATGGAACGAACACAAACAAAAAGTGAAGAAAC
AHSV1_Z26316        GCGTTGATATCGACCCAAATAATGGTACGTGGAGTAAGCATAAAGCGGAAGTTAAGAAGT
AHSV3_U01832        GCGTTGATATCGACCCAAATAATGGTACGTGGAGTAAGCATAAAGCGGAAGTTAAGAAGT
AHSV3_DQ868772      GTGTTGATATCGACCCAAATAATGGTACGTGGAGTAAGCATAAAACGGAAGTTAAGAAGA
AHSV7_AY163330      GCGTAGATATTAATCCTAACAATTCGACGTGGATCGAGCATAAGAAGAAGATGGCGGAAA
                       *  *  *  * ** **          ****   *  *          *    *

AHSV8_DQ868775      GTTTGAATGAAGCGCAGAAGAAGAATCGAAATGTTCCTCATTTAATGCTTGTTGATGGAG
AHSV8_AY163333      GTTTGAATGAAGCGCAGAAGAAGAATGGAGGTGTTCCTCATTTAATGCTTGTTGATGGAG
AHSV5_AY163331      AGTTGGATGAGGCGCAAAAGAAGAACAATAACGAACCGTATAAAGTAATGGTTGATGGGG
AHSV6_DQ868774      AAATGAATCGTGCGCAGCAAGGCAACAATAATAAACCTCTAAAGATTCAGATTGATGGAG
AHSV6_NC_005996     AAATGAATCGTGCGCAACAAGGCAACAATAATAAGCCTCTAAAGGTTCAAATCGATGGTG
AHSV9_DQ868776      GGATGCAGAAAGAACAGAGCGCGAATTCTAATTCACCCTTAAAGATCCAGGTTGATGGGG
AHSV4_EU046574      AATTGAAGAAAGCGCAAACCGAAAATGGAGGACAACCATGCCAAGTGTCGATCGATGGAG
AHSV4_DQ868773      AATTGAAGAAAGCGCTAACCGAAAATGGAGGACAACCATGCCAAGTGTCGATCGATGGAG
AHSV4_D26570        GATTAAAGAAAGCGCAAAACGAAAATGGAGGACAGCCATGCCAAGTGTCGATCGATGGGG
AHSV1_FJ011108      AGTTGAAAAAGAAACAAGAGGAGAACCGAGCG---CCTCTAACCGTTCAAATTGATGGGG
AHSV1_AY163329      AGTTGAAAAAGAAACAAGAGGAGAACCGAGCG---CCTCTAACCGTTCAAATTGATGGCG
AHSV2_AY163332      AATTAGAAGAAAAACAGGAAGAGAATGGGTCT---CCCATGTCCGTTCAGATCGATGGGG
AHSV1_Z26316        TTTTGAACGAGGAACAAAAGAAGAATGAAAATAAACCATTAAAGGTGTTGATTGATGGCG
AHSV3_U01832        TTTTGAACGAGGAACAAAAGAAGAATGAAAATAAACCATTAAAGGTGTTGATTGATGGCG
AHSV3_DQ868772      TTTTGGACGAGGAACAAAAGAAGAATGAAAACAAACCATTAAAGGTGTTGATTGATGGCG
AHSV7_AY163330      AGCTAAAGGATGAGCAACAGAAAAACCAAAATCGTCCGATGCTAGTGCCGATTGATGGTG
                       *  *       *        **          **        *     * ***** *

AHSV8_DQ868775      TGAATATTATGACAGATAAAAAATATGGGACCGTTCAAAATTGGGTTGATTGGGTCGTGA
AHSV8_AY163333      TGAATATTATGACAGATAAAAAATACGGGACCGTTCAAAATTGGGTTGATTGGGTCGTGA
AHSV5_AY163331      TTAACATCATGACGAATAAGAAGTACGGATCCGTTGAAAACTGGGTCGATTGGGTTGTAA
AHSV6_DQ868774      TTAGCATACTCACGAATGAAAAGTATGGTACCGTTGGTCATTGGGTTGATTGGGTTGTTG
AHSV6_NC_005996     TTAGCATACTCACGAGTGAAAAATATGGTACCGTTGGTCATTGGGTCGATTGGGTTGTTG
AHSV9_DQ868776      TCAGTTTATCAACCGGTGAATTCTACGGTACGGTTGAACATTGGATAGATTGGGTTGTTG
AHSV4_EU046574      TAAACGTCTTGACTAACGTAGATTACGGTACGGTTAATCATTGGATAGATTGGGTAACAG
AHSV4_DQ868773      TAAACGTCTTGACTAACGTAGATTACGGTACGGTTAATCATTGGATAGATTGGGTAACAG
AHSV4_D26570        TAAACATCTTAACTAACGTGGATTATGGAACGGTTAATCATTGGATCGATTGGGTAACAG
AHSV1_FJ011108      TGCACATACGTACGGATGAAACCTATGGAACAGTAGATCATTGGGTTGAATGGGTCGTTG
AHSV1_AY163329      TGCACATACGTACGGATGAAACCTATGGAACAGTAGATCATTGGGTTGAATGGGTCGTTG
AHSV2_AY163332      TTTATATCCGAACAGATGTGCCGTATGGAACGGTTGATCATTGGGTTGATTGGGTTGTTG
AHSV1_Z26316        CGTACATATCAACCGATGCTGAGTACGGAACCGTAGCGCATTGGGTGGATTGGGTCGTTG
AHSV3_U01832        CGTACATATCAACCGATGCTGAGTACGGAACCGTAGCGCATTGGGTGGATTGGGTCGTTG
AHSV3_DQ868772      CGTACATATCAACTGATGCTGAGTACGGAACCGTAGCGCATTGGGTGGACTGGGTCGTGG
AHSV7_AY163330      TCTACGTCTCCACTAGTGTTGAGTATGGTACCGTTACACATTGGGTTGATTGGGTCGTTG
                                  *    **          ** **  * **     * *** * ** *****

AHSV8_DQ868775      ATTATATCATGTTATCGCACGTGAAACGTTTAGTTAAGGATTATAAATTTAAAAGATTGC
AHSV8_AY163333      ATTATATCATGTTATCGCACGTGAAACGTTTAGTTAAGGATTATAAATTTAAAAAACTGC
AHSV5_AY163331      ATTATATTATGTTATCGCACGTCAAAAGGCTGGTTAAGGACTATAAATTTAAGAGGCTCA
AHSV6_DQ868774      ACTTAATTATGTTAGCGCAGGTGAAGATGTTAATAAAAGAATATAAGTTTAAAAGATTGA
AHSV6_NC_005996     ATTTGATTATGTTGGCGCAGGTGAAAATGTTAATAAAAGAATATAAGTTTAAAAGATTAA
AHSV9_DQ868776      ATTTAATTATGTTAGCACAGGTGAAACGATTAATCAAGGAGTACAAGTTTGTAAGATTGG
AHSV4_EU046574      ATATAATTATGGTTGTACAAACTAAACGTTTGGTGAAAGAGTATGCATTTAAAAAACTAA
AHSV4_DQ868773      ATATAATTATGGTTGTACAAACTAAACGTTTGGTGAAAGAGTATGCATTTAAAAAACTAA
AHSV4_D26570        ATATGATTATGGTCGTACAGACTAAACGATTAGTAAAAGAGTATGCGTTTAAAAAACTGA
AHSV1_FJ011108      ACACAATCATGCTTAGAGAGACTGAAAAAATGATTAAGGATTATCGGTTCAAAAAACTTA
AHSV1_AY163329      ACACAATCATGCTTAGAGAGACTGAAAAAATGATTAAGGATTATCGGTTCAAAAAACTTA
AHSV2_AY163332      ACACAATCATGCTTAAGGAGACTGACAAAATGATAAAGGATTACGAATTTAAGAAATTAA
AHSV1_Z26316        ATATCGTAATGATGACTCAAGTTAGCCGCATGATAAAAGAATATAATTTTATTAGATTAA
AHSV3_U01832        ATATCGTAATGATGACTCAAGTTAGCCGCATGATAAAAGAATATAATTTTATTAGATTAA
AHSV3_DQ868772      ATATCATAATGACGACTCAAGTTAGCCGCATGATAAAGGAATATAATTTTATTAGATTGA
AHSV7_AY163330      ATATTATAATGACAACGCAGGTTGAAAGAATGATAAAAGAGTATGATTTTAAACGATTGG
                    *     * ***      *           *  * ** ** **    **       *

AHSV8_DQ868775      AACCGGATAATTTGATGTCAGGCATGAATAAGCTTGTAGGTGCGTTGAGGTGTTACGCAT
AHSV8_AY163333      AACCGGATAATTTGATGTCAGGCATGAATAAGCTTGTAGGTGCGTTGAGGTGTTACGCAT
AHSV5_AY163331      AGCCGGATAATTTGATGTCCGGGATGAATAAGCTAGTCGGCGCACTAAGATGTTATGCGT
AHSV6_DQ868774      ATAGCCAAAATTTGATGTCGGGTATGAATAAACTGGTCGGTGCACTGAGATGTTATGCTT
AHSV6_NC_005996     ATAGCCAAAATTTGATGTCGGGTATGAACAAACTGGTTGGTGCGCTGAGATGTTATGCTT
AHSV9_DQ868776      AAACCTCGAACTTAATGGCGGGAATGAATAAATTAGTTGGAGCCTTGCGCTGCTACGCCT
AHSV4_EU046574      AGAGCGAAAACTTACTTGCTGGAATGAATAGTTTAGTTGGGGTATTAAGATGTTATATGT
AHSV4_DQ868773      AGAGCGAAAACTTACTTGCTGGAATGAATAGTTTAGTTGGGGTATTAAGATGTTATATGT
AHSV4_D26570        AGAGCGAAAACCTACTTGCCGGAATGAATGGTTTAGTTGGGATATTAAGATGTTATATGT
AHSV1_FJ011108      AGCGTGAGGAGCTGATCTCTGGAATGAATAAATTAGAGGATGGCTTACGATGTATCGTGT
AHSV1_AY163329      AGCGTGAGGAGCTGATCTCTGGAATGAATAAATTAGAGGATGGCTTACGATGTATCGTGT
AHSV2_AY163332      AACGTGAAGAATTGATAGCTGGTATGAACAAGTTGGAGGATGGTTTGAGATGCATTGTTT
AHSV1_Z26316        AAAAGGATCAGCTAATCAGCGGTATGAATAAGTTGGAGGATGGGGTAAAATGCTACGCGT
AHSV3_U01832        AAAAGGATCAGCTAATCAGCGGTATGAATAAGTTGGAGGATGGGGTAAAATGCTACGCGT
AHSV3_DQ868772      AAAAGGATCAGCTAATCAGCGGTATGAATAAATTAGAGGATGGGGTAAAATGCTACGCGT
AHSV7_AY163330      AGAGAAAACAGCTAATTAGTGGTATGAACAAGCTCGAGGATGGAGTTAAATGTTATGCTT
                    *       *  *  *    ** *****     * *  *      *    **       *
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AHSV8_DQ868775      ATTGCTTAATCCTTGCGTTGTATGATCATTTCGGAGCGGAAATCGAAGGATTTAGGAAGG
AHSV8_AY163333      ATTGCCTAATCCTTGCGTTGTATGATCATTTCGGAGCGGAAATTGAAGGATTTAGGAAGG
AHSV5_AY163331      ATTGTTTGATACTCGCGTTATATGATTATTTCGGGGAGGATATCGAAGGGTTTAAGAAAG
AHSV6_DQ868774      ACTGCTTGATTCTCGCTTTGTATGACTATTATGGTCAAGATATCGAGGGCTTCAAGAAGG
AHSV6_NC_005996     ACTGCTTGATTCTTGCTTTATATGACTATTATGGTCAAGATATTGAGGGCTTCAAGAAGG
AHSV9_DQ868776      ATTGCTTGATCTTAGCTCTCTATGATTTTTATGGCGCAGATATTGAAGGTTTTGAGAAGG
AHSV4_EU046574      ATTGCTTAGCTTTAGCGATCTATGATTTTTATGAAGGGACTATTGATGGTTTTAAGAAAG
AHSV4_DQ868773      ATTGCTTAGCTTTAGCGATCTATGATTTTTATGAAGGGACTATTGATGGTTTTAAGAAAG
AHSV4_D26570        ATTGTTTAGCTTTAGCGATTTATGATTTTTATGAAGGAATTATTGATGGTTTTAAGAAAG
AHSV1_FJ011108      ATTGCTTAATACTTGCTCTATACGATTATTATGAAGGAGATATCGAGGGTTTTAAGAAGG
AHSV1_AY163329      ATTGCTTAATACTTGCTCTATACGATTATTATGAAGGAGATATCGAGGGTTTTAAGAAGG
AHSV2_AY163332      ATTGCTTGATTTTAGCGTTATATGATTATTATGGGGCTGAGATTGAGGGATTCAAAAAGG
AHSV1_Z26316        ATTGTTTGATCCTCGCACTGTACGATTTTCATGGGCGTGACGTAGACGGCTTTGCGCAGG
AHSV3_U01832        ATTGTTTGATCCTCGCACTGTACGATTTTCATGGGCGTGACGTAGACGGCTTTGCGCAGG
AHSV3_DQ868772      ATTGTTTGATCCTCGCATTATATGATTTTCATGGGCGTGAACTAGACGGCTTTGCGCAGG
AHSV7_AY163330      ATTGCTTGATTTTGGCATTGTACGATTATTATGGAGATGCGATCGAGGGTTTCGCGCAAG
                    * **  *     * **  * ** **   *   *         * ** ** **     * *

AHSV8_DQ868775      GGACTAACGCAGCTTCGATAGTTGAAACAGTTTCCCAGATGTTTCCAAATTTCAGAAAGG
AHSV8_AY163333      GGACTAACGCAGCTTCGATAGTTGAAACAGTTTCCCAAATGTTTCCAAATTTCAGAAAGG
AHSV5_AY163331      GAACGAACGCGGCTTCAATTGTAGAAACAGTGTCCCAAATGTTCCCGCAGTTTAGGAAAG
AHSV6_DQ868774      GATCAAATTCTTCCGCGATATTAGAGACGGTTATTCAAATGTTTCCGAATTTTAAACAGG
AHSV6_NC_005996     GATCAAATTCGTCTGCGATATTAGAAACGGTTATTCAGATGTTTCCGAATTTTAAACAGG
AHSV9_DQ868776      GTTCGAACTCATCCGCGATTGTGGAGACGGTTGTACAGATGTTCCCGAACTTCAAGCAGG
AHSV4_EU046574      GCTCGAATGCTTCCGCTATCATTGAAACTGTCGCGCAGATGTTTCCGGACTTTCGCAGAG
AHSV4_DQ868773      GCTCGAATGCTTCCGCTATCATTGAAACTGTCGCGCAGATGTTTCCGGACTTTCGCAGAG
AHSV4_D26570        GTTCAAACGCTTCCGCTATCATTGAAACCGTCGCGCGAATGTTTCCTGATTTTCGCTCTG
AHSV1_FJ011108      GGACTATTGCTTCGTCAATAGTTGAGACCGTTTCACAGATGTTTCCAAATTTTCGAAGCG
AHSV1_AY163329      GGACTATTGCTTCGTCAATAGTTGAGACCGTTTCACAGATGTTTCCAAATTTTCGAAGCG
AHSV2_AY163332      GGACAAACGCTTCATCTATAGTTGAGACTGTATCACAAATGTTCCCAAACTTCCGTGGAG
AHSV1_Z26316        GGACGAGAACCGCAGCTATTGTTGAGACCGTCGCGCGAATGTTTCCTGATTTTCGCTCTG
AHSV3_U01832        GGACGAGAACCGCAGCTATTGTTGAGACCGTCGCGCGAATGTTTCCTGATTTTCGCTCTG
AHSV3_DQ868772      GAACGAGAACCGCGGCTATTGTCGAGACCGTCGCACGAATGTTTCCCGATTTTCGCTCCG
AHSV7_AY163330      GAACGCGTGCGGGTTCGATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAG
                    *  *     *     * **  * ** **  *    *  ***** **  * **       *

AHSV8_DQ868775      AAGTTTCGGAAACTTTTGGCATCGATCTCAAAACAAAAGAGATTAAG-CACGAACTTTTC
AHSV8_AY163333      AGGTTTCGGAAACTTTTGGCATCGATCTCAAAACAAAAGAGATTAAG-CACGAACTTTTC
AHSV5_AY163331      AAGTTTCAGAGACCTTTGGAATCACTCTGAAATACAAAAGACGTCAAA-TATGAGTTGTTT
AHSV6_DQ868774      AAATTCAAGCTAATTTCGGGATAAATTTGAACATTAAAGATAAAAAA-CAATCGTTATTC
AHSV6_NC_005996     AAATTCAAGCTAATTTCGGAATAAATTTGAATATTAAAGACAAAAAAACAATCGCTATTC
AHSV9_DQ868776      AGATTCAAGCTAATTTTGGTATAAATCTAAATATCAAGGATAAAAAA-CAGGCGTTGTTC
AHSV4_EU046574      AACTTGTCGAAAAATTCGGTATAGATTTAAGGATGAAGGAAATCACG-CGTGAGTTGTTT
AHSV4_DQ868773      AGCTTGTCGAAAAATTCGGTATAGATTTAAGGATGAAGGAAATCACG-CGTGAGTTGTTT
AHSV4_D26570        AGCTCGTTGAAAAGTTCGGTATAGATCTAAGGATGAAGGAAATCACG-CGTGAGTTGTTT
AHSV1_FJ011108      ATATAATAGATAAATTCGGTATTTCTCTGAAAGTTAAAACTGAGGCC-GAAGAATTGTTT
AHSV1_AY163329      ATATAATAGATAAATTCGGTATTTCTCTGAAAGTTAAAACTGAGGCC-GAAGAATTGTTT
AHSV2_AY163332      ATATCATCGACAAATTTGGCATACAGCTGAAGGTGAAAAGGGAAGCT-GAGGAGTTGTTC
AHSV1_Z26316        AGGTTTCGGAAAAATTCGGTATTGATTTAGCGGTGTCAGAGGAATCA-GATGAACTATTC
AHSV3_U01832        AGGTTTCGGAAAAATTCGGTATTGATTTAGCGGTGTCAGAGGAATCA-GATGAACTATTC
AHSV3_DQ868772      AGGTTTCGGAAAAATTCGGCATTGATTTAGCGGTGTCAGAGGAATCA-GATGAATTATTC
AHSV7_AY163330      ACGTAGCGGAAAAGTTTGGTATTAAACTGACTATAAAGGACGAATCA-GAAGAGTTGTTC
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AHSV8_DQ868775      AAAGCTCAAAATATGAATGTTAAAGCGGCTGATGTTGGAGATTACGGATACAAGTTTCAG
AHSV8_AY163333      AAAGCTCAAAATATGAATGTTAAAGCGGCTGATGTTGGAGATTACGGATACAAGTTTCAG
AHSV5_AY163331      ATCGCTAGAGACATGAGTGCGAAAGAGGCTCAGTTCGGTGAGGTTGGGTACAAATTCCAG
AHSV6_DQ868774      GTTGAGCGAACGATGCATTCGGATTTCTCATCAAATGAAGAGTACGGATATAAATTTGTA
AHSV6_NC_005996     GTCGAGCGA-CGATGCATTCGGACTTCTCATCGAATGAAGAGTACGGATATAAATTTGTA
AHSV9_DQ868776      GTCAGGATGGATATGGATTCAGAGTTTAGTGAAGATGAGCAGAAGGGATATATGTTTGAG
AHSV4_EU046574      GTTGGTAAGAGCATGACGTCAAAATTTATGGAGGAAGGTGAATATGGATATAAGTTCGCC
AHSV4_DQ868773      GTTGGTAAGAGCATGACGTCAAAATTTATGGAGGAAGGTGAATATGGATATAAGTTCGCC
AHSV4_D26570        GTCGGTAAGAGCATGACGTCAAAATTTATGGAAGAAGGTGAATATGGATATAAGTTCACC
AHSV1_FJ011108      CTACCCAAGAATATGAAGTCATCTATGAATGTCGGGGAAGAGAGAGGATACAAGTATAAG
AHSV1_AY163329      CTACCCAAGAATATGAAGTCATCTATGAATGTCGGGGAAGAGAGAGGATACAAGTATAAG
AHSV2_AY163332      CTTCCGAAGGATATGGTATCATCTTTTCTAGAGGATGGCGAAGAGGGATATAAATATCAA
AHSV1_Z26316        GTAAAGAAGACGATGGTCTCGAGTTTCTCTGACTCTGGGGAGATGGGTTACAAATTCATA
AHSV3_U01832        GTAAAGAAGACGATGGTCTCGAGTTTCTCTGACTCTGGGGAGATGGGTTACAAATTCATA
AHSV3_DQ868772      GTGAAGAAAACGATGGTTTCGAGTTTCTCTGACTCTGGGGAGATGGGTTATAAATTCATA
AHSV7_AY163330      GTTCAAAAAGATATGAATTCGGAGTTTTTGGATGAGGGAGAGATGGGCTATAAATTCGTG
                                                ***                 *   *    ** ** *  *

AHSV8_DQ868775      TACGGGTGGACTCGAACAGCGGAGCAAGTAATGAGTGATTATGGAGAAATATTGACTGAA
AHSV8_AY163333      TACGGGTGGACTCGAACAGCGGAGCAGGTAATGAGTGATTACGGAGAAATATTGACTGAA
AHSV5_AY163331      TATGGGTGGAGAAAAACGGATCAAAAAGTCATGAGCGATTACGCTGACATTTTGAGCGAA
AHSV6_DQ868774      TTTGGGTGGGCGGCAAGAGGTGAAGAAGTGTTGAGTAATTATGGAGATATTCTCTCAGAT
AHSV6_NC_005996     TTTGGATGGGCGGCAAGAGGTGAAGAAGTGTTGAGTAATTATGGAGATGTTCTCTCAGAT
AHSV9_DQ868776      TATGGATGGGCGAAGAGAGAAGAGCAAATATGGTCAAATTATGGTGACATATTAACTGAC
AHSV4_EU046574      TATGGATGGCGTAGGGATGGCTTCGCGGTGATGGAAGATTACGGAGAAATTTTGACAGAA
AHSV4_DQ868773      TATGGATGGCGTAGGGATGGCTTCGCGGTGATGGAAGATTACGGAGAAATTTTGACAGAA
AHSV4_D26570        TATGGGTGGCGTAGGGATGGCTTCGCGGTGATGGAAGATTACGGAGAGATTTTGACAGAG
AHSV1_FJ011108      TTTGGGTGGAAGGATAATGAGGAGAGAGTGATGAGTGATTACGGGGAAATTTTGACTGAA
AHSV1_AY163329      TTTGGGTGGAAGGATAATGAGGAGAGAGTGATGAGTGATTACGGGGAAATTTTGACTGAA
AHSV2_AY163332      TACGGTTGGAAAGATAATGAAGAATTAGTGGCAAGCGATTATGGTGAAATTTTGACTCAG
AHSV1_Z26316        TTTGGATGGAGGAAAACTGATTTCAAGGTTGAGACTGATTATGGAGAGATAGTTTCTGAT
AHSV3_U01832        TTTGGATGGAGGAAAACTGATTTCAAGGTTGAGACTGATTATGGAGAGATAGTTTCTGAT
AHSV3_DQ868772      TTCGGATGGAGGAAAACTGATTTCAAGGTTGAGACTGATTATGGAGAGATAGTTTCTGAC
AHSV7_AY163330      TTCGGGTGGAAAAATACAGATTTTAAAGTACAGAGCAATTATGGCGAGATCGTTTCAGAG
                    *  ** ***        *        *       **** *  **  *  *    *

AHSV8_DQ868775      GAAATTGAAACGCTATATCAATCCATTTTAGCAGGCAAAGAATGGGAAAAGGTTTCTGAT
AHSV8_AY163333      GAAATTGAAACGTTATATCAATCAATTTTAGCAGGCAAAGAATGGGGAAAGGTTTCTGAT
AHSV5_AY163331      AAGGTTGAAGCATTATATCAAGCACTACTTTCTGGAAGGAAGTGGAGCGATATAGCTGAT
AHSV6_DQ868774      GAGGTTGAAGAATTATTCACAAAACTTAGGAAGAAAGAGCATTGGGATAAGGTTGTGGAA
AHSV6_NC_005996     GAGGTTGAAGAATTATTCACGAAACTTAGGAAGAAAGAGCATTGGGATAAGGTTGTGGAA
AHSV9_DQ868776      TTGGTTGAACAGCTTTATAAGAGCATTATGAATCATGAGGAGTGGGAGAAAATCGTTGAT
AHSV4_EU046574      AAAGTGGAGGACCTATATAAGGGTGTACTTTTAGGACGAAAGTGGGAGGATGAGGTTGAT
AHSV4_DQ868773      AAAGTGGAGGACCTATATAAGGGTGTACTTTTAGGACGAAAGTGGGAGGATGAGGTTGAT
AHSV4_D26570        AAAGTGGAGGATTTATATAAGAGTGTACTTTTAGAGCGAAAGTGGGAAGATGAGGTCGAT
AHSV1_FJ011108      TCGGTGGAGATCCTATTTAAGAAGCTTCTTAAGGGAGAAAAGTGGAAGATTATCGTTGAT
AHSV1_AY163329      TCGGTGGAGATCCTATTTAAGAAGCTTCTTAAGGGAGAAAAGTGGAAGATTATCGTTGAT
AHSV2_AY163332      TCAGTACAAATTCTGTTCGAAGGATTGATGAGAGGGGAGAAGTGGACTTCGATTATAGAT
AHSV1_Z26316        GAAGTCCATCGGTTATATCAAGCAATTTTGGATGGCAAGGAATGGAGTAAAGAGGTTGAT
AHSV3_U01832        GAAGTCCATCGGTTATATCAAGCAATTTTGGATGGCAAGGAATGGAGTAAAGAGGTTGAT
AHSV3_DQ868772      GAAGTTCATCGGTTATATCAAGCAATTTTGGATGGCAAAGAATGGAGTAAAGAGGTTGAT
AHSV7_AY163330      GAAGTTGAAAGGTTATTCAAAGTGATTTTGGAAGGTAAGGAGTGGTCAAATGAGGTAGAG
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AHSV8_DQ868775      GAAACGGATGTTTATTTTATCGATGATTTATTTTCCTCAACGCCGGACAAGGTATTCAGA
AHSV8_AY163333      GAAACGGATGTTTATTTCATCGATGATTTATTTTCCTCAACGCCGGACAAGGTATTCAGA
AHSV5_AY163331      GATACGGAAGAGTATTTTATAGATGATTTGTACGTTAATAAGCCAGATAGGGTCTTTGAG
AHSV6_DQ868774      GATCCAGAGTCCTATTTCGTAGATGAGCTTTATCAAAAGAATCCAGCGGAAGTATTTTTT
AHSV6_NC_005996     GATCCAGAATCTTATTTCATAGATGAGCTTTATCAAAAGAATCCGGCGGAAGTGTTTTAT
AHSV9_DQ868776      GACCCAGAAAAGTATTTCTATGATGATCTTTTTAACGCATCACCAGAGACGGCTTTTATT
AHSV4_EU046574      GATCCAGAGAGTTATTTTTATGATGATCTTTATACTAATGAGCCCCACAGAGTGTTTCTA
AHSV4_DQ868773      GATCCAGAGAGTTATTTTTATGATGATCTTTATACTAATGAGCCCCACAGAGTGTTTCTA
AHSV4_D26570        GATCCAGAGAGCTATTTCTATGATGATCTTTATACCAATGAGCCCCACAGAGTATTTCTA
AHSV1_FJ011108      GATCCGCAAACTTACTTCGAAGATGACTTGTTCGTTGATCGGGCGAACAAGATTTTCTCT
AHSV1_AY163329      GATCCGCAAACTTACTTCGAAGATGACTTGTTCGTTGATCGGGCGAACAAGATTTTCTCT
AHSV2_AY163332      GATCCTCAGTCCTACTTTGAGGATGATATATTTGCGGGAAAAGCTAATAGGATGTTTTTG
AHSV1_Z26316        GACCCTGAAAAATACTTCGTTGATGATTTATATAATAGATGCCCGGAGTCAATATATGTC
AHSV3_U01832        GACCCTGAAAAATACTTCGTTGATGATTTATATAATAGATGCCCGGAGTCAATATATGTC
AHSV3_DQ868772      GATCCAGAAAAATACTTCGTTGATGATTTATATAATAGATGCCCGGAGTCAATATACGTT
AHSV7_AY163330      GACCCGGAGGATTATTTCGTTGATAACTTGTTTAATAAAACACCGGATGCAGTGTTTGAG
                    **  *  *    ** **    *** *  * *           *          *

AHSV8_DQ868775      CGTGTTGGA------CTAGATTCTCAAAATAATATCAAGATTGAGGGTAAAATGAA----
AHSV8_AY163333      CGTGTTGGA------CTAGATTCTCAAAATAATATCAAGATTGAGGGTAAAATGAA----
AHSV5_AY163331      AGAGCTGGG------TTAGATCCGGAGAGACACATCAAGGTTAAAGGTGTGATGAA----
AHSV6_DQ868774      AGTGCAGGT------TATGATACAGATCAAAATGTGGTAATCGATGGGAAAATGAC----
AHSV6_NC_005996     AGCGCAGGC------TATGATACAGATCAAAATGTGGTAATCGATGGGAAAATGAC----
AHSV9_DQ868776      AGCAAAGGT------TATGATCCTGACAATAATATCGTTATTGAGGGAAAGGTAGG----
AHSV4_EU046574      AGCGCAGGA------AAGGATGTGGATAATAATATCACGCTTCGATCGATTTCGCA----
AHSV4_DQ868773      AGCGCAGGA------AAGGATGTGGATAATAATATCACGCTTCGATCGATTTCGCA----
AHSV4_D26570        AGCGCAGGA------AAGGATGTGGACAATAATATTACACTTCGATCGATTTCGCA----
AHSV1_FJ011108      AGGGGTGGACAGACGGTGAATCAGCTGATAAGTATCAAGGTGAATGCGCAATCAAATAAC
AHSV1_AY163329      AGGGGTGGACAGACGGCGAATCAGCTGATAAGTATCAAGGTGAATGCGCAATCAAATAAC
AHSV2_AY163332      AGAGGCGGAGAGACGGTTGAACGGCATGTGAAATTGAAGGTTAACGCTCAGACAGAGAAC
AHSV1_Z26316        AGGAACGGA------GTTGATCCTAATAATAAGATAATGATTAAGAAGCGAGGTTTAGTT
AHSV3_U01832        AGGAACGGA------GTTGATCCTAATAATAAGATAATGATTAAGAAGCGAGGTTTAGTT
AHSV3_DQ868772      AGGAACGGA------GTTGATCCTGATAATAAGATAATGATTAAGAAGCGAGGTTTAGTT
AHSV7_AY163330      AGAGACGGA------ATGGACGGAAGCAACAGGATTATCGTTAAGAATAAAACTACACTT
                     *    **          *             *     *

AHSV8_DQ868775      --TGAGTTGACTACATACTTCTCAAAACGTTTTGTTACCTATTGGTATAAAATTACAAAG
AHSV8_AY163333      --TGAGTTGACTACATACTTCTCAAAACGTTTTGTTACCCATTGGTATAAAATTACAAGG
AHSV5_AY163331      --TGAGCTGACGACGTACTTCTCAAAACGTTTCATTTCTTATTGGTATAAAATAACTAAG
AHSV6_DQ868774      --AGAGGGGGTCACGTACTTCTCTAAAAGATTCGTATCGTACTGGTATCGTGTTGAGAAA
AHSV6_NC_005996     --AGAGGGAGTCACGTACTTCTCTAAAAGATTTGTTTCGTATTGGTATCGTGTTGAGAAA
AHSV9_DQ868776      --TCAAGATGTTACTTATTTTTCGAAACGGTTTGTATCATATTGGTATCGAGTTCGACAG
AHSV4_EU046574      --GGCGGAAACCACGTATCTATCGAAGCGTTTCGTATCATATTGGTATAGAATATCACAA
AHSV4_DQ868773      --GGCGGAAACCACGTATCTATCGAAACGTTTCGTATCATATTGGTATAGAATATCACAA
AHSV4_D26570        --GGCGGAGACTACGTATTTGTCGAAACGTTTCGTATCGTATTGGTATAGAATATCGCAA
AHSV1_FJ011108      GTTGAGGGTACGACCTATTTTTCGAAAAGATTTGTTTCTTATTGGTTCAGAATAGAACAT
AHSV1_AY163329      GTTGAGGGTACGACCTATTTTTCGAAAAGATTTGTTTCTTATTGGTTCAGAATAGAACAT
AHSV2_AY163332      GTTGAGGGCATCACATACTTTTCAAAAAGATTTGTATCTTATTGGTTCAGAATTGAACGC
AHSV1_Z26316        GGGGAGAGCCAGCG-CATTTTTCTGCGAGATTTGTCTCATATTGGTATGAATTTCAAAAA
AHSV3_U01832        GGGGAGAGCCAGCG-CATTTTTCTGCGAGATTTGTCTCATATTGGTATGAATTTCAAAAA
AHSV3_DQ868772      GGGGAGGGCCAACGGCATTTTTTCCGCGAGATTCGTCTCATATTGGTATGAATTTCAAAAA
AHSV7_AY163330      CGAGAGGGACAACGCCATTTTTCAGCTCGTTTTGTTTCGTATTGGTATACGTTTGAGAAG
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AHSV8_DQ868775      GTGGAGAAGAAAGATTTACTCATTGT-GAATGATATCTACGATGAAAAAAC-GGAA---T
AHSV8_AY163333      GTGGAGAAGAAAGATTTACTCATTGT-GAATGATATCTACGATGAAAAAACCGGAA---T
AHSV5_AY163331      GTTGAAGCAAGGAATCTATTGACTTT-AACTGATATAGGGGGGGATGCAAA-AAAG---T
AHSV6_DQ868774      AT---AACAACGAAG---CATCTCG--AGTTTTTA---AATGAAGAGGGTCGAAAA---G
AHSV6_NC_005996     AT---AACAACGAAG---CACCTTG--AGTTTTTA---ACTGAAGAGAATCGAAAA---G
AHSV9_DQ868776      GTTCAGACCTCAAAG---GGTGCTG--AGAGGAGA---TCGATTGAGGATGTCAAA---T
AHSV4_EU046574      GTTGAAGTAACGAAGGCGCGTAATG--AAGTTCTGGACATGAATGAGAAACAGAAGCCGT
AHSV4_DQ868773      GTTGAAGTAACGAAGGCGCGTAATG--AAGTTCTGGACATGAATGAGAAACAGAAGCCGT
AHSV4_D26570        ATTGAAGTGACAAAGGCGCGTAATG--AGGTTCCGGATATGAATGAGAAACAGAAGCCGT
AHSV1_FJ011108      TTTTCGATTACGACAGC-AAAAAAGCGAACTGACATTCGAGATAAAAAGACTGAA----T
AHSV1_AY163329      TTTTCGATTACGACAGC-AAAAAAGCGAACTGACATTCGAGATAAAAAGACTGAA----T
AHSV2_AY163332      ACAACGCACGTAAAGGG-AGGGGGAAGAGTAGATATTCGGGATCGAAAAACAGGG----T
AHSV1_Z26316        GTTACTATTGAGGCTGAGTTCGAAGCGATTGCACGCACGTGGCGAACATATACAG----T
AHSV3_U01832        GTTACTATTGAGGCTGAGTTCGAAGCGATTGCACGCACGTGGCGAACATATACAG----T
AHSV3_DQ868772      GTTACTATTAAGGCTGA-TTCGAAGCGATTGGACGCACGTGGCGAACATACACAG----T
AHSV7_AY163330      GTGGAGGTTG-GTCGGGCGATGGCGAGAATCGATATTCATGATCGAGAGACCAAG----T

AHSV8_DQ868775      ATCAACAGTTTGATCC-GGATGATTTCAAGCCAA-TGGTTATTGGAGAGATGGGTGTTCA
AHSV8_AY163333      ATCAGCAGTTTTATCCCGGATGATTTCAAGCCAAATGGTTATTGGAGAGATGGGTGTTCA
AHSV5_AY163331      ATACACAGTTTGATCC-TGATGATTTTAAACCAA-TGGCTGTGGCTGAGCTGGGAGCACA
AHSV6_DQ868774      TTGCGCAATTTGATTT-CGAGGATTATAAACCGA-TGGCTATTGGAGAGATGGGGATTCA
AHSV6_NC_005996     TCGCGCAGTTTGATTT-TGAGGATTATAAACCGA-TGGCTATTGGAGAGATGGGGATTCA
AHSV9_DQ868776      ATAGGGAGTTTGATAT-AGAAAGTTTTAAGCCTT-ACGCGATTGGTGAGATCGGTATTCA
AHSV4_EU046574      ATTTTGAATTTGAATA-TGATGATTTCAAACCCT-GTTCAATTGGAGAGTTGGGGATCCA
AHSV4_DQ868773      ATTTTGAATTTGAATA-TGATGATTTCAAACCCT-GTTCAATTGGAGAGTTGGGGATCCA
AHSV4_D26570        ATTTTGAATTTGAGTA-TGACGATTTTAAGCCTT-GCTCGATTGGAGAGTTGGGGATCCA
AHSV1_FJ011108      ATAACGAGTTTGACTT-CGAAGACTTCAAGCCGG-CATGTATTGGAGAATTGGGGATACA
AHSV1_AY163329      ATAACGAGTTTGACTT-CGAAGACTTCAAGCCGG-CATGTATTGGAGAATTGGGGATACA
AHSV2_AY163332      ACCAGCAGTTCGATGT-AGAAGATTTTAAACCTG-CAAGTGTAGGGGAACTTGGATTTCA
AHSV1_Z26316        ATCATGAGATAGATGT-TGAAGATTTCAAACCTT-GCGCGATTGCGGAACTTGGATTACA
AHSV3_U01832        ATCATGAGATAGATGT-TGAAGATTTCAAACCTT-GCGCGATTGCGGAACTTGGATTACA
AHSV3_DQ868772      ATCATGAGATAGATGT-TGAAGATTTCAAACCCT-GCGCGATTGCGGAACTTGGATTACA
AHSV7_AY163330      TTTCCGAGTTCGATGT-CGATGACTATAAACCAT-GTTCAGTGGCGGAAATGGGGCTACA
                          *  *  *    **    *  ** **        * *  **  * **    **

AHSV8_DQ868775      TGCTTCGACATATATATATCAAAACTTGATTTTAGGTAGAAATAGGGGTGAGCGGATTGT
AHSV8_AY163333      CGCTTCAACATATATATATCAAAACTTGATTTTAGGTAGAAATAGGGGTGAGCGGATTGT
AHSV5_AY163331      CGCGTCGACGTATGTTTATCAGAATTTGATTTTAGGGAGGAACAGAGGCGAGAAAATTGG
AHSV6_DQ868774      TGCATCGACATATAAGTATGAGTCGTTGCTTCTTGGAAAGAATAGAGGGCAAAAAGTGAA
AHSV6_NC_005996     TGCGTCGACATATAAGTATGAGTCGTTGCTCCTTGGGAAAAATAGAGGGCAAAAAGTGAA
AHSV9_DQ868776      CGCATCAACTTACAAATATCTGGATCTATTAGCGGGGCGTAATCGCGGAGAGAAAGTGAA
AHSV4_EU046574      TGCATCCACATATATATATCAGAACCTACTGGTCGGACGTAATAGAGGTGAGGAAATACT
AHSV4_DQ868773      TGCATCCACATATATATATCAGAACCTACTGGTCGGACGTAATAGAGGTGAGGAAATACT
AHSV4_D26570        TGCATCGACATACATATATCAGAACCTCCTCGTTGGACGTAATAGGGGCGAGGAGATACT
AHSV1_FJ011108      CGCGTCGACATATATCTACCAGGATTTGTTGGTCGGGAAGAGCAGAGGGGAACGAGTGAA
AHSV1_AY163329      CGCGTCGACATATATCTACCAGGATTTGTTGGTCGGGAAGAGCAGAGGGGAACGAGTGAA
AHSV2_AY163332      CGCGTCAACATATATTTATCAAGATTTACTTGTGGGAGCAAATAGAGGCGAGCGTGTGAA
AHSV1_Z26316        TTGCTCAACCTATATTTATCAAGATTTGCTCGTTGGCGCGAACAGAGGTGAATATGTGAA
AHSV3_U01832        TTGCTCAACCTATATTTATCAAGATTTGCTCGTTGGCGCGAACAGAGGTGAATATGTGAA
AHSV3_DQ868772      TTGCTCAACCTATATTTATCAAGATTTGCTCGTTGGCGCGAACAGAGGTGAATATGTGAA
AHSV7_AY163330      TTCATCAACTTATATCTATCAAGATTTGTTGATAGGCCCAAATAGGGGAGAGCATGTGAT
                        ** ** **    **       *  *    **    *   * **  *    *
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AHSV8_DQ868775   TGATAGCAAGGAGATCGTGTGG-TATGATTTGTCGTTGACCAATTTCGGCCTGGTGCGTT
AHSV8_AY163333   TGATAGCAAAGAGATCGTGTGG-TATGATTTGTCGTTGACCAATTTCGACCTGGTGCGTT
AHSV5_AY163331   TGATGCGAAGGAGATCGTTTGG-TATGATTTATCTTTGACTAATTTTGGATGTTCTAGAT
AHSV6_DQ868774   AGACTCCATTGCTTT-GTGTAACTATGATCTTGCACTTACGAACTTCGAAGTCTCGAGGC
AHSV6_NC_005996  TGATTCCATCGCTCT-GTGTAACTACGATCTTGCACTTACGAATTTCGGGGTTTCGAGGC
AHSV9_DQ868776   GGATTCGCAAGCGCTCGTGTGG-TATGATTTCGCTTTAACTAACTACACGCTAGTGCGAC
AHSV4_EU046574   TGATTCGAAAGAGCTCGTCTGGATG-GATATGTCACTTTTAAATTTTGGAGCGGTCAGAT
AHSV4_DQ868773   TGATTCGAAAGAGCTCGTCTGGATG-GATATGTCACTTTTAAATTTTGGAGCGGTCAGAT
AHSV4_D26570     TGATTCGAAGGAGCTTGTCTGGATG-GATATGTCGCTATTAAATTTTGGAGCGGTCAGAC
AHSV1_FJ011108   AGACGCGAAGGAATTGGTGTGGATG-GATCTTTCGCTGGCGAATTTTGGGTGTTCGCGCT
AHSV1_AY163329   AGACGCGAAGGAATTGGTGTGGATG-GATCTTTCGCTGGCGAATTTTGGGTGTTCGCGCT
AHSV2_AY163332   GGACGCGAAAGAGTTAGTGTGGATG-GATTTATCTCTTACCAATTTCGGGTTTGTGCGAA
AHSV1_Z26316     GGATGCGAAGGAGCTCGTCTGGTTC-GATATCGCTAACACAAACTTCAACATCACGCGTC
AHSV3_U01832     GGATGCGAAGGAGCTCGTCTGGTTC-GATATCGCTAACACAAACTTCAACATCACGCGTC
AHSV3_DQ868772   GGATGCGAAGGAGCTCGTTTGGTTC-GATATCGCTAACACAAACTACAACATCACGCGTC
AHSV7_AY163330   TGATGCAAAAGAATTGGTTTGGTAT-GATATTGCGTTAACAAATTATGGAACAACGCGCT
                  **       *   * ** *      *** *  *       ** *            *

AHSV8_DQ868775   CGCAGAATCAATGCTGGATTGGATCAATTTCAAATTTTGAGTTAAGTATGCGATATCACA
AHSV8_AY163333   CGCAGAATCAATGTTGGATTGGATCAATTTCAAATTTTGAGTTAAGTATGCGATATCACA
AHSV5_AY163331   CGTTAGATTCTTGTTGGGTGGGGTCAGTTGCTAGATCTGAGCTGAATTTGCGGTTCCACC
AHSV6_DQ868774   GTCAGGATTGCTGCTGGATCTCATCGTGTAGCGCCATTGAGCTTTCGATGAGAGCCAACA
AHSV6_NC_005996  GTCAGGACTGTTGCTGGATCTCATCGTGTAGTGCCATTGAGCTTTCGATGAGAGCCAACA
AHSV9_DQ868776   CACAAGATAGATGTTGGATAATGTCGTGTACTGATTGTGAATACACTTTGAGATTTGCAA
AHSV4_EU046574   CTCACGATAGGTGCTGGATCTCCTCAAGCGTCGCGATTGAGGTGAATTTACGTCATGCAC
AHSV4_DQ868773   CTCACGATAGGTGCTGGATCTCCTCAAGCGTCGCGATTGAGGTGAATTTACGTCATGCAC
AHSV4_D26570     CTCACGATAGGTGTTGGATTTCTTCAAGCGTCGCGATTGAGGTAAATCTACGTCACGCAC
AHSV1_FJ011108   GCTATGATCGATGTTGGCCAGCTTCCTGCGTTGAAGCGGAGATCTCCTTGAGGTACTATT
AHSV1_AY163329   GCTATGATCGATGTTGGCCAGCTTCCTGCGTTGAAGCGGAGATCTCCTTGAGGTACTATT
AHSV2_AY163332   GCTACAATAGATGTTGGATTGCGGCCTTTGTGGAGGCGGAGATTTCGCTCAGATTTTATC
AHSV1_Z26316     CTTTTGATAGATGCTGGCCTTCTTCTTGCGCGGAGGCTGAGTTATCCTTGAGGTTTCATC
AHSV3_U01832     CTTTTGATAGATGCTGGCCTTCTTCTTGCGCGGAGGCTGAGTTATCCTTGAGGTTTCATC
AHSV3_DQ868772   CCTTTGATAGATGCTGGCCTTCTTCTTGCGCGGAGGCTGAGTTATCCTTGAGATTTCATC
AHSV7_AY163330   ATTTTGATCAGTGTTGGCCATCCTCATGTTCCACCACGGAATTATCAATGAGATATTTTT
                       *    ** ***       *             **        *  *

AHSV8_DQ868775   TCATAACCGAAATTTTCCAGAGGT---ATAGAGTGGATTCAGCACATAAGTCTTACCACG
AHSV8_AY163333   TCATAACCGAAATTTTCCAGAGGT---ATAGAGTGGATTCAGCACATAAGTCTTACCACG
AHSV5_AY163331   TTATATCCGCAATATTTGAACGAT---ATCAGCATGATGCACGACGGAGCTCCTTTTATG
AHSV6_DQ868774   TCACTATCGCAATTTTTAGGAGA----ATCGAAGACAGAAGATATGAGAGTTTCGCGAAA
AHSV6_NC_005996  TCATTATCGCTATTTTTAGGAGA----ATCGAAGACAAGAGATATGAGAATTTCGCGAAA
AHSV9_DQ868776   CGATCACTATGATATTCGAACGTTT--ATCTGAGGAAGCTGATCTTTCT-TATCATGATA
AHSV4_EU046574   TAATAGTTAGGATTTTTCACGCTTTGACATGATGTCGGAAAGAGAAACGTTTT-CAACC
AHSV4_DQ868773   TAATAGTTAGGATTTTTCACGCTTTGACATGATGTCGGAAAGAGAAACGTTTT-CAACC
AHSV4_D26570     TGATAGTTAGGATTTTTCACGCTTTGACATGATGTCGGAGAGAGAAGCGTATT-CAATG
AHSV1_FJ011108   TAGTCACATCTATTTTCGCTAGAT---ACCTTAACCGTGAAGGATTGAG-CTTTTCAAAG
AHSV1_AY163329   TAGTCACATCTATTTTCGCTAGAT---ACCTTAACCGTGAAGGATTGAG-CTTTTCAAAG
AHSV2_AY163332   TCATAACGTCGATCTTTTGTAGAT---ATTTTACTGGCGATAGGAAGAG-TTTCGCTAAG
AHSV1_Z26316     TGATTACCAAGATCTTCACGAGAT---ATCGA---GGCGAAAGAACTTC-GTTTGTCGAT
AHSV3_U01832     TGATTACCAAGATCTTCACGAGAT---ATCGA---GGCGAAAGAACTTC-GTTTGTCGAT
AHSV3_DQ868772   TGATTACCAAGATCTTCACGAGAT---ATCGA---GGCGAAAGAACTTC-GTTTGTCGAT
AHSV7_AY163330   TGATTACTGAAATCTTTCAGAGAT---ATAGGACTGACGATAGATCATC-TTTTGCGGAT
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AHSV8_DQ868775   AAATTATCAGCGGACTGACTAAGAAAGACGTG----ATTCTTTTCCCTAGTTATAAACAT
AHSV8_AY163333   AAATTATCAGCGGACTGACTAAGAAAGACGTG----ATTCTTTTCCCTAGTTATAAACAT
AHSV5_AY163331   AAATTATCTTCGATCTGCCCTCTAAGAAAGAG----AGGATCTTCCCGAGTTACAAACAT
AHSV6_DQ868774   ATACTATCCGGACTTTCGCAGCAGCAAGACTT------ATACTTTCCTACGTATAAGCAT
AHSV6_NC_005996  ATATTATCCGGACTCACACAGCAGCAAGACCT------ATATTTCCCTACGTATAAGCAT
AHSV9_DQ868776   -TCTTATTGAAGGTCCGCGAATATCCGATCCA------ATCATTCGCTTCGTATAAACAT
AHSV4_EU046574   ATTTTAGAAAAAGTCATGGAGGATGTGAAAGAGTTGAGATTTTTCCCGACATATCGTCAT
AHSV4_DQ868773   ATTTTAGAAAAAGTCATGGAGGATGTGAAAGAGTTGAGATTTTTCCCGACATATCGTCAT
AHSV4_D26570     ATTTTGGAAAAAGTCATGGAGGATGTGAAGGAGCTGAGATTTTTCCCAACATATCGCCAT
AHSV1_FJ011108   ATTTTGATCTCTCTCA--AAGATTTTTCGGATAGATTATGGTTTCCAA-CGTATAAACAT
AHSV1_AY163329   ATTTTGATCTCTCTCA--AAGATTTTTCGGATAGATTATGGTTTCCAA-CGTATAAACAT
AHSV2_AY163332   ATACTAGATGGAGTCA--AGTCATTGAAAGAAAGATTATGGTTCCCTA-CCTACAAACAT
AHSV1_Z26316     ATAATTAATGAGTTGAGTGAGCATGGCTACGTGAAACACAATTTCCCGTCATATAAGCAT
AHSV3_U01832     ATAATTAATGAGTTGAGTGAGCATGGCTACGTGAAACACAATTTCCCGTCATATAAGCAT
AHSV3_DQ868772   ATAATTAATGAGTTGAGTGAGCGAGGCTACGTGAAACACAATTTCCCGTCATATAAGCAT
AHSV7_AY163330   ATCTTGGAGAGGATGCGTAAGAACGGGTATCCCCGTCGCAATTTTCTGACGTATAAACAT
                     *                                     *        **    ***

AHSV8_DQ868775   TATTATGTTAGAGTTATACAAGATGTATTTCAAGATTCGCAAAAGGTAGATGTTTTAGAT
AHSV8_AY163333   TATTATGTTCGAGTTATACAAGATGTATTTCAAGATTCGCAAAAGGTAGATGTTTTAGAT
AHSV5_AY163331   TATTACGTCGCTTTGCTACAGAATATCTTTAATGATACTCAACGATTAGAGGTAATGGAC
AHSV6_DQ868774   TATTATCTGTTTGTATTACAAAAGGTGTTACGAGATGAGAGGAGGATAGATCAGAATAGA
AHSV6_NC_005996  TATTATCTGTTTGTGTTACAAAAGGTATTACGAGATGAGAGGAGGATAGATCTAAATAGA
AHSV9_DQ868776   TTCTATGTTCGCGTCTTGCAGCACGTTTTTAGAGATAACCAAGAAATCGACGTATTAGAG
AHSV4_EU046574   TATTATTTGGAAACTCTCCAACGTGTCTTTAACGATGAGAGACGCTTAGAAGTTGATGAC
AHSV4_DQ868773   TATTATTTGGAAACTCTCCAACGTGTCTTTAACGATGAGAGACGCTTAGAAGTTGATGAC
AHSV4_D26570     TATTATTTGGAAACTCTCCAACGTGTCTTTAACGATGAGAGACGTTTGGAGGTTGATGAC
AHSV1_FJ011108   TTTTATGTTGCAGTTGTACAAAAAGTGCTCCGCGATGATAGACGCTTAGATTATGTACTC
AHSV1_AY163329   TTTTATGTTGCAGTTGTACAAAAAGTGCTCCGCGATGATAGACGCTTAGATTATGTACTC
AHSV2_AY163332   TACTATGTTGCCGTCATTCAGAAGATTTACTCAGATGATAGGAATCTAGATAGGAATGAA
AHSV1_Z26316     TATTACCTCTCTGTTATTCAGACGGTTTTTGAGGATCAAAGAGCGATCGATCCGCTTGAC
AHSV3_U01832     TATTACCTCTCTGTTATTCAGACGGTTTTTGAGGATCAAAGAGCGATCGATCCGCTTGAC
AHSV3_DQ868772   TATTACCTCTCTGTCATTCAGACGGTTTTTGAGGATCAAAGAGCAATCGATCCGCTTGAC
AHSV7_AY163330   TATTATGTCGCTGTCATTCAAGAGGTATTTGGTGATCAAAGACCGATCGATGTGTATAGT
                 *  **  *        * **     *       ***          * **

AHSV8_DQ868775   TTCTGTTTGAGGATTGCCAACCCAGAGACGCGCTTATCCACGCTGCTGAAGATTCAGGGC
AHSV8_AY163333   TTCTGTTTGAGGATTGCGAACCCTGAGACGCGCTTATCTACGCTGCTGAAGATTCAGGGT
AHSV5_AY163331   TATTGCGAGAGACTGATGAATCCGGAAACGAGGATGTCAGCCCTCCTATCACTTCAGGGG
AHSV6_DQ868774   ATGTGCACAGAGCTGTTTGATATACAGAGGCGAAGAGGTATCCTCCTTTCGTTTACCACG
AHSV6_NC_005996  ATATGCACCGAGTTGTTTGATACACAGAGGCGAAGAGGTATTCTCCTTTCGTTTACCGCG
AHSV9_DQ868776   TTCTGTACTAGGATGCTCGATCCGAGGACACGTGAAGCGGGGTTAAATAAGTTCTCAAGG
AHSV4_EU046574   TTTTATATGAGGTTATATGATGTGCAGACAAGGGAGCGGGCACTAAATACTTTCACGGAT
AHSV4_DQ868773   TTTTATATGAGGTTATATGATGTGCAGACAAGGGAGCAGGCACTAAATACTTTCACGGAT
AHSV4_D26570     TTTTATATGAAGTTATATAATGTGCAAACAAGGGAGCAGGCACTAAATACTTTCACGGAC
AHSV1_FJ011108   TTTTGTTCTAGGGTCTCGGCAATCACAACCCGGCGTGCGACGTTAATGGAATTTAGTACC
AHSV1_AY163329   TTTTGTTCTAGGGTCTCGGCAATCACAACCCGGCGTGCGACGTTAATGGAATTTAGTACC
AHSV2_AY163332   TTTTGTAATAGAGTCATGAATATCATGACAAGACGGTCAGTACTCAATGAGTTTGAGAGG
AHSV1_Z26316     TTTTGTGCAATGATTTCACGGAATGAGACGCGAGAGAGCACGCTCAAGGGATTTAGTATG
AHSV3_U01832     TTTTGTGCAATGATTTCACGGAATGAGACGCGAGAGAGCACGCTCAAGGGATTTAGTATG
AHSV3_DQ868772   TTTTGTGCAATGATTTCACGGAATGAGACGCGAGAGAGCACGCTCAAGGGATTTAGTATG
AHSV7_AY163330   TTTTGTACCGATATCTTTAAGAAAGAGAGGCGCCGAGGGGTTTTGAGTATGTTTCCTACC
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AHSV8_DQ868775      TTCAGAGCATGTGTGGAATCTGAGTTCCTTCTCCCAACACTTCATCTGAATTTTTTGATT
AHSV8_AY163333      TTCAGAGCATGTGTGGAATCTGAGTTCCTTCTCCCAACACTTCACCTGAATTTTTTGATT
AHSV5_AY163331      TTCAGAAACTGTGTCGAGTCGGAGTTCGTGGCCCCAACGTTGAAGATGAATGCACTTCTT
AHSV6_DQ868774      TTGAGATTTTGGAACGATTCGGAGTTTCTAGGTGATACGTTAATGATGAATTTTCTACTA
AHSV6_NC_005996     TTGAGATTTTGGAATGATTCAGAATTTTTGGGTGATGCGTTAATGATGAATTTTCTACAT
AHSV9_DQ868776      TTTAGACAATGGAGAGAATCGGAATTCTTGATAGATGCTCTTAAAATGAATTTCCTTTTG
AHSV4_EU046574      TTTCACAGGTGTGTTGAGTCGGAACTGCTCTTACCGACACTTAAACTTAACTTTCTGCTG
AHSV4_DQ868773      TTTCACAGGTGTGTTGAGTCGGAACTGCTCTTACCGACACTTAAACTTAACTTTCTGCTG
AHSV4_D26570        TTTCAAAGGTGTGTTCAGTCAGAATTGCTCTTACCAACGCTTAAGCTCAATTTTTTGTTG
AHSV1_FJ011108      TTTAAACAGATGGTGGGATCAACACGTTTATTGGATACGCTATTCTTAAATTTTCTACTT
AHSV1_AY163329      TTTAAACAGATGGTGGGATCAACACGTTTATTGGATACGCTATTCTTAAATTTTCTACTT
AHSV2_AY163332      TTTAGAAGAGCTATTGAGTCGCCAAAGCTGATTGACACTTTGTCGCTTAATTTTCTCCTT
AHSV1_Z26316        TTTACTGCAATTGTTAAAAGTGAGAGACTGATAGATACCCTGTTCTTGAATTTTCTTGTTA
AHSV3_U01832        TTTACTGCAATTGTTAAAAGTGAGAGACTGATAGATACCCTGTTCTTGAATTTCTTGTTA
AHSV3_DQ868772      TTTGCTGCAATTGTTAAGAGTGAGAGACTGATAGATACCTTGTTTTTGAATTTCTTGTTA
AHSV7_AY163330      TTTTCTACCTTAATCAAGAGCGAAAAATTGATCGATGCTTTATTTCTTAATTTCTTCTTG
                    **                          *       *  *     * **     *

AHSV8_DQ868775      TGGCTGCTGATTGACATGGAAAATGGGGACATAAATTACTCGAAGAAAAGACTTCCTTTA
AHSV8_AY163333      TGGTTGCTGATTGACATGGAAAACGGGGACATAAATTACTCGAAGAAAAGGCTTCCTTTA
AHSV5_AY163331      TGGGTTTTAGCGGATATGGAGAATATTGATATAAACTACTCGAACAAGCGGATGCCGCTG
AHSV6_DQ868774      TGGGTGGTTTTTGAGATGGAAAACATCGATGTGGATTATGGGAAAAAGTGGCATCCGCTG
AHSV6_NC_005996     AGGGTGGTTTTCGAGATGGAAAACGTCGATGTGGATTATGGGAAGAAGTGGCATCCGCTA
AHSV9_DQ868776      TGGGTGGTCTTCGAACTGGAAAATATAGATGTTGATTATAGTAAGAAAAGGCATCCTTTA
AHSV4_EU046574      TGGATTGTTTTTGAAATGGAAAATGTTGAAGTGAACGCGGCGTACAAGCGTCATCCGCTT
AHSV4_DQ868773      TGGATTGTTTTTGAAATGGAAAATGTTGAAGTGAACGCGGCGTACAAGCGTCATCCGCTT
AHSV4_D26570        TGGATCGTTTTTGAGATGGAAAATGTTGAAGTGAATGCAGCGGACAAGCGTCATCCGCTT
AHSV1_FJ011108      TGGATCATTTTCGAACAGGAGAATATAGATGTTGATTTCGCAAATAAGTGGCATCCTCTA
AHSV1_AY163329      TGGATCATTTTCGAACAGGAGAATATAGATGTTGATTTCGCAAATAAGTGGCATCCTCTA
AHSV2_AY163332      TGGATAATTTTCGAACAAGAGAACATAGATGTTAATTTTGCGGATAAGAGGCATCCACTA
AHSV1_Z26316        TGGATTGTCTTTGAAATGGAGAATGTTGATGTGAGCGCTGCTAATAAGAGACATCCATTA
AHSV3_U01832        TGGATTGTCTTTGAAATGGAGAATGTTGATGTGAGCGCTGCTAATAAGAGACATCCATTA
AHSV3_DQ868772      TGGATTGTTTTTGAAATGGAGAATGTTGATGTGAGCGCTGCTAATAAGAGACATCCATTA
AHSV7_AY163330      TGGGTTGTTTTCGAGATGGAAAACGTTGACGTAAGTTTTGCAAATAAGCGTCATCCTTTA
                     ** *  *    **    ** **    **  *          * **  *    **  *

AHSV8_DQ868775      TTGATTTCTACGACGAACGGTTTGAGGGTGATGGCGGTCGACGCATTCAATAACATGATT
AHSV8_AY163333      TTGATTTCTACGACGAAGGGTTTGAGGGTGATGGCGGTCGACGCATTCAATAACTTGATT
AHSV5_AY163331      TTGTTATCAACTGAGAAGGGCCTACGTGTGATTTCTATTGATATGTTTAACGGAATGCTG
AHSV6_DQ868774      CTAGTATCGTCAGAAAAGGGGCTTAGAGTGATTGCGGTTGACGTTTTTAATTCAATGATG
AHSV6_NC_005996     CTAGTATCGTCAGAGAAGGGGCTTAGAGTGATCGCGGTTGATGTTTTTAACTCAATGATG
AHSV9_DQ868776      CTAATATCTACCGACAAAGGGTTAAGAGTAGTCTCAGTTGATCTTTTTAATTCTATGCTT
AHSV4_EU046574      TTAATCTCAACTGCCAAAGGGTTAAGGGTTATCGGCGTTGATATTTTCAACTCACAGCTT
AHSV4_DQ868773      TTAATCTCAACTGCCAAAGGGTTAAGGGTTATCGGCGTTGATATTTTCAACTCACAGCTT
AHSV4_D26570        TTAATTTCAACTGCCAAAGGATTAGGGGTTATTGGCGTTGACATTTTCAACTCGCAGCTT
AHSV1_FJ011108      TTAATTTCTACAGAAAAGGGCTTGAGAGTTATTGCGGTTGATGTTTTTAATAGTTCACTT
AHSV1_AY163329      TTAATTTCTACAGAAAAGGGCTTGAGAGTTATTGCGGTTGATGTTTTTAATAGTTCACTT
AHSV2_AY163332      TTAATATCAACGACTAAAGGTTTGAGAGTAATCCCGATTGACGTTTTCAATAGTTCACTA
AHSV1_Z26316        TTAATATCGCATGAAAAAGGATTACGTTTAATTGGCGTAGATTTGTTTAATGGCGCGCTT
AHSV3_U01832        TTAATATCGCATGAAAAAGGATTACGTTTAATTGGCGTAGATTTGTTTAATGGCGCGCTT
AHSV3_DQ868772      TTAATATCGCATGAGAAAGGATTACGTTTAATTGGCGTAGATTTGTTCAATGGCGCGCTT
AHSV7_AY163330      TTGATATCGCACGATAAGGGTTTGCGATTGATTGGTGTAGATTTGTTCAATAGTGCGTTA
                     *  * **       ** **  *  *  *  *     * **    ** **       *
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AHSV8_DQ868775      GCCATGTCTTATAGCGGTTGGCTCCCGTATTTAGAGAGAATATGTCACGAAACAAAGCAG
AHSV8_AY163333      GCCATGTCTTATAGCGGTTGGCTCCCGTATTTAGAGAGAATATGTCACGAAACAAAGCAG
AHSV5_AY163331      GGTGTTTCTTATAGCGGCTGGATCCCATACTTAGAGAGGATCTGCAGTGAAGTAAATCTA
AHSV6_DQ868774      GGAGTATCAACGAGTGGTTGGCTTCCTTATGTTGAACGCATCTGTTCTGAGTCCGATATG
AHSV6_NC_005996     GGAGTGTCGACAAGTGGCTGGCTTCCTTATGTTGAACGCATTCTGAGTCCGAGTCTGATATG
AHSV9_DQ868776      AGCGTTTCCCTGAGTGGGTGGATCCCATATGTTGAAAGAGTGTGTGAACGCTCGGAAGCT
AHSV4_EU046574      TCGATATCAATGAGCGGATGGATTCCGTATGTCGAACGGATGTGCGCGGAGAGTAAAGTT
AHSV4_DQ868773      TCGATATCAATGAGCGGATGGATTCCGTATGTCGAACGGATGTGCGCGGAGAGTAAAGTT
AHSV4_D26570        TCGATATCAATGAGCGGGTGGATTCCGTACGTCGAAAGGATGTGCGCGGAGAGTAAAGTT
AHSV1_FJ011108      ACGTTATCAACGAGCGGATGGCTGCCATATTTAGAGCGGATTTGCTCTGAGTCCGCGATG
AHSV1_AY163329      ACGTTATCAACGAGCGGATGGCTGCCATATTTAGAGCGGATTTGCTCTGAGTCCGCGATG
AHSV2_AY163332      GCTCTTTCTCCAAGTGGTTGGATCCCTTACGTTGAGAGGATATGTGCAGAGGCTAAGGAA
AHSV1_Z26316        TCGATTTCCACGGGGGGGGTGGATTCCGTATCTAGAGAGGATATGTTCAGAGGAGAAAGCT
AHSV3_U01832        TCGATTTCCACGGGGGGGTGGATTCCGTATCTAGAGAGGATATGTTCAGAGGAGAAAGCT
AHSV3_DQ868772      TCGATTTCCACGGGGGGGTGGATTCCGTATCTAGAGAGGATATGTTCAGAGGAGAAAGCT
AHSV7_AY163330      TCCATTTCAATGGGTGGATGGATCCCTTATGTTGAAAGGATATGCCATGCAGATCATGCA
                        * **    * ** *** * ** **  * **  *  * **

AHSV8_DQ868775      CGGACGAGACTCAATGCTGATGAATTAAAATTAAAGAAGTGGTTTCTGAATTACGTTACG
AHSV8_AY163333      CGGACGAGACTCAATGCTGATGAATTAAAATTAAAGAAGTGGTTTCTGAATTACGTTACG
AHSV5_AY163331      CAGAGACGCTTAAGAGCGGATGAGCTAAAATTGAGGAAGTGGTTTATCAGCTACTACGCT
AHSV6_DQ868774      CGCAGACGACTGAATGCGGATGAATTAGAGTTGAAGCGCTGGTTCTTTGATTATTACGCG
AHSV6_NC_005996     CGCAGGCGATTGAATGCAGATGAACTAGAGTTAAAACGGTGGTTCTTTGACTACTATGCG
AHSV9_DQ868776      AAAAGAAGATTGAATGCGGATGAATTGAAACTAAAGAACTGGTTTATTGCGTATTATGTC
AHSV4_EU046574      CAAACAAAATTGACGGCTGATGAGCTGAAATTGAAGAGGTGGTTCATCTCATATTATACG
AHSV4_DQ868773      CAAACAAAATTGACGGCTGATGAGCTGAAATTGAAGAGGTGGTTCATCTCATATTATACG
AHSV4_D26570        CAAACAAAATTGACGGCCGATGAACTAAAGTTGAAGAGGTGGTTTATTTCATATTATACG
AHSV1_FJ011108      GATCGAGCTCTCACCGCTGACGAAATAAATCTTAAACGATGGTTTGTTGATTACTACATG
AHSV1_AY163329      GATCGAGCTCTCACCGCTGACGAAATAAATCTTAAACGATGGTTTGTTGATTACTACATG
AHSV2_AY163332      AACCGTACGCTGAGTTCCGATGAATTGAGAATTAAAACCTGGTTCGTAGAGTACTATCTA
AHSV1_Z26316        CAGAGAAGGTTGAACGCGGATGAACTGAAAATAAAAAGTTGGTTTTTAACGTATTATATG
AHSV3_U01832        CAGAGAAGGTTGAACGCGGATGAACTGAAAATAAAAAGTTGGTTTTTAACGTATTATATG
AHSV3_DQ868772      CAGAGAAGGTTGAACGCGGATGAACTTAAAATAAAAAGTTGGTTTTTAACGTATTATATG
AHSV7_AY163330      GCAAGAAAATTGAATGCGGACGAACTGAAAATAAAGAGGTGGTTTATAGATTATTATATG
                                  * *   * ** **  *    * *    *****  *   **

AHSV8_DQ868775      AAATACGAAGTTGAGAGAAGGGCGGAGCCGCGTATGAGTTTCAAAATGGAGGGCATAACA
AHSV8_AY163333      AAATACGAAGTTGAAAGAAGGGCGGAGCCGCGTATGAGTTTCAAAATGGAGGGCATAACA
AHSV5_AY163331      ACCTATGAAGTTGAGAGACGTGCTGAGCCAAGAATGAGTTTTAAAATGGAGGGAATCTCC
AHSV6_DQ868774      ACATTATTGCTTGAAAGGAGGGGGGAGCCTCGGTTGAGCTTCAAGTATGAAGGATTGACC
AHSV6_NC_005996     ACATTACCGCTCGAAAGGAGAGGAGAGCCTCGGTTGAGCTTTAAGTATGAAGGATTGACC
AHSV9_DQ868776      ACTTTGCCTCTGTTAAGGCGTGCGGAACCAAGGATGAGCTTTAAGTATGAGGGAATTACA
AHSV4_EU046574      ACGTTGAAATTGGACCGCAGAGCGGAGCCACGTATGAGTTTCAAATTTGAGGGGTTGAGT
AHSV4_DQ868773      ACGTTGAAATTGGACCGCAGAGCGGAGCCACGTATGAGTTTCAAATTTGAGGGGTTGAGT
AHSV4_D26570        ACGTTGAAGTTGGACCGCAGAGCGGAGCCACGTATGAGTTTCAAATTCGAAGGGTTAAGT
AHSV1_FJ011108      GAGCTGAAGTTGGAGAGACGAGCGGAACCTCGAATGAGTTTCAAGAGCGAGGCTTTGATC
AHSV1_AY163329      GAGCTGAAGTTGGAGAGACGAGCGGAACCTCGAATGAGTTTCAAGAGCGAGGCTTTGATC
AHSV2_AY163332      AATATAAACTTAGAGAGGAGGGCGGAGCCTAGGATGAGTTTCAAGAGTGAAGCGCTGATC
AHSV1_Z26316        AATCTTTCGTTAGAAAGGAGAGCGGAGCCGCGTATGAGCTTTAAGTTCGAAGGGTTAACC
AHSV3_U01832        AATCTTTCGTTAGAAAGGAGAGCGGAGCCGCGTATGAGCTTTAAGTTCGAAGGGTTAACC
AHSV3_DQ868772      AATCTTTCGTTAGAAAGAAGAGCGGAGCCGCGTATGAGCTTTAAGTTCGAAGGATTAACC
AHSV7_AY163330      GATCTAAGTTTAGATCGACGAGCAGAGCCGAGAATGAGTTTCAAATATGAAGGACTAGCG
                                  *    *  * *  ** **  *  **** ** **    ** *   *
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AHSV8_DQ868775      ACGTGGATTGGATCAAACTGCGGTGGGGTTCAGGATTACATCTTACATTTGATACCATCC
AHSV8_AY163333      ACGTGGATTGGATCAAACTGCGGTGGGGTTCAGGATTACATCTTACATTTGATACCATCC
AHSV5_AY163331      ACCTGGATTGGTTCAAACTGTGGGGGCGTCCAAGATTACGTGTTACATTTGATTCCTTCA
AHSV6_DQ868774      ACGTGGATTGGCTCAAATTGTGGAGGAGTGCGCGATTACGTTGTACAATTGCTTCCCATG
AHSV6_NC_005996     ACGTGGATTGGCTCAAATTGTGGAGGGGTGCGGGATTACGTTGTGCAATTGCTTCCTATG
AHSV9_DQ868776      ACGTGGATTGGTTCAAATTGCGGGGGGGTACGAGACTATCTGATTCAGATGCTCCCAGCA
AHSV4_EU046574      ACATGGATCGGTTCGAACTGCGGAGGTGTTAGGGATTACGTAATACAGATGCTTCCTACC
AHSV4_DQ868773      ACATGGATCGGTTCGAACTGCGGAGGTGTTAGGGATTACGTAATACAGATGCTTCCTACC
AHSV4_D26570        ACATGGATCGGTTCGAACTGCGGAGGTGTTAGGGATTACGTGATACAGATGCTTCCTACC
AHSV1_FJ011108      ACGTGGATCGGGTCAAATTGTGGAGGAGTGACCGATTATGTCGTACAACTTCTACCCGTT
AHSV1_AY163329      ACGTGGATCGGGTCAAATTGTGGAGGAGTGACCGATTATGTCGTACAACTTCTACCCGTT
AHSV2_AY163332      ACGTGGATCGGATCGAATTGTGGTGGAGTGACTGATTATGTGGTTCAGCTTTTGCCTGTT
AHSV1_Z26316        ACTTGGATCGGCTCAAATTGTGGTGGAGTTCGCGATTATGTCGTCCAAGCTCTACCGATG
AHSV3_U01832        ACTTGGATCGGCTCAAATTGTGGTGGAGTTCGCGATTATGTCGTCCAAGCTCTACCGATG
AHSV3_DQ868772      ACTTGGATTGGCTCAAATTGTGGTGGAGTTCGCGATTATGTCGTCCAAGCTCTACCGATG
AHSV7_AY163330      ACGTGGGTTGGATCGAACTGCGGTGGTGTTAGAGACTATATTATCCAGGAGTTGCCAATG
                    ** *** * ** ** ** ** ** ** **    ** **  *  * **     * **

AHSV8_DQ868775      CGGAAACCGAAGCCTGGATTATTATTTTTGATTTATACAGACGCAGGGGATGTAGATTGG
AHSV8_AY163333      CGGAAACCAAAACCTGGATTATTATTTTTGATTTATACGGACGCAGGGGATGTAGATTGG
AHSV5_AY163331      CGAAAACCAAAGCCTGGACTTCTTTTTCTGATATATGCGGATGATGGTGACGTTGACTGG
AHSV6_DQ868774      CGTAAACCAAAGCCAGGGCTTCTCTGCATCGCTTACGGTGATGACGTCAATGTACAGTGG
AHSV6_NC_005996     CGCAAATCAAAGCCAGGGCTTCTCTGCATCGCTTACGGTGATGACGTCAATGTACAGTGG
AHSV9_DQ868776      AGAAAACCGAAACCGGGCGTTTTAATCTTAGCTCATGGTGCGGAGATTAACGTAGCGTGG
AHSV4_EU046574      AGAAAACCTAAACCGGGAGCTTTGATGGTGGTATACGCGCGGGATTCGAGAATCGAGTGG
AHSV4_DQ868773      AGAAAACCTAAACCGGGAGCTTTGATGGTGGTATACGCGCGGGATTCGAGAATCGAGTGG
AHSV4_D26570        AGAAAACCGAAACCGGGAGCCTTGATGGTAGCGTACGCGCGGGATTCGAGAATTGAATGG
AHSV1_FJ011108      CGAAAGCCGAAACCGGGGTTATTGGTTGTCGTATATTCGGAGGATGGTGGGGAAAAATGG
AHSV1_AY163329      CGAAAGCCGAAACCGGGGTTATTGGTTCTCGTATATTCGGAGGATGGTGGGGAAAAATGG
AHSV2_AY163332      CGGAAACCGAAACCGGGCCTGCTAGTGGTTGTTTATTCGGAGGACGGGAGTGGGAAATGG
AHSV1_Z26316        CGGAAACCAAAGCCTGGTTTATTGATGATAATTTATGGAGACGATGGGGACGCGCGTTGG
AHSV3_U01832        CGGAAACCAAAGCCTGGTTTATTGATGATAATTTATGGAGACGATGGGGACGCGCGTTGG
AHSV3_DQ868772      CGGAAACCAAAGCCTGGTTTATTGATGGTAATTTATGGAGACGATGGGGACGCGCGTTGG
AHSV7_AY163330      CGAAAGCCAAAACCTGGACTTTTAATATTAGTGTATGGAGAAGATGGAGATCCAAAATGG
                     * **  * ** ** **     *     *     *       *             ***

AHSV8_DQ868775      GTAACACGGATGCTTTACGATGTGTGTCGACTAGAGGGTAGCTTGGGCTTCATTTTAATC
AHSV8_AY163333      GTAACACGGATGCTTTACGATGTGTGTCGATTAGAGGGTAGCTTGGGCTTCATTTTAATC
AHSV5_AY163331      GTAGCGAACATGCTATCAGATGTGATTGGTTCGGAGGGTAGTTTAGGATTCATCTTTATT
AHSV6_DQ868774      GTTGAACATGAGTTAAGGGATTTTTTGACACATGAGGGCTCGCTCGGCTTAGTGGTAATT
AHSV6_NC_005996     GTTGAACATGAGTTAAGAGATTTTTTGATGCATGAGGGCTCACTCGGCTTAGTGGTAATT
AHSV9_DQ868776      TTGAACCATGCGCTACGTGATATACTTTCGCTAGAGGGATCGTTAGGGATAATTATTGTG
AHSV4_EU046574      ATCGAAGCAGAGCTATCACAGTGGCTGCAAATGGAAGGTTCGCTTGGTTTGATCCTCGTT
AHSV4_DQ868773      ATCGAAGCAGAGCTATCACAGTGGCTGCAAATGGAAGGTTCGCTTGGTTTGATCCTCGTT
AHSV4_D26570        ATCGAGGCGGAGCTATCACAGTGGCTGCAAATGGAGGGTTCGCTCGGATTGATTCTCGTT
AHSV1_FJ011108      GCAGAGTGGGCTTTACGTGACTACCTCGAAATTGATGGTAGTTTGGGTTTAGTATTCATT
AHSV1_AY163329      GCAGAGTGGGCTTTACGTGACTACCTCGAAATTGATGGTAGTTTGGGTTTAGTATTCATT
AHSV2_AY163332      GCAGAATGGGCGCTACGCGATTTTTTAGACGTTGAAGGTAGCTTAGGATTAATTTTCATA
AHSV1_Z26316        GTAGAGTGGGCAATGAAGAATTTTACAGCGGTTGATGGATCGTTGGGCTTCATTTATATC
AHSV3_U01832        GTAGAGTGGGCAATGAAGAATTTTACAGCGGTTGATGGATCGTTGGGCTTCATTTATATC
AHSV3_DQ868772      GTAGAATGGGCAATGAAGAATTTTACAGCGGTTGACGGATCGTTGGGCTTCATTTATATC
AHSV7_AY163330      GTTGAGTGGGCGATTAAGGATTTTACTCAAATTGAAGGCTCTCTTGGCTTTATATACATT
                              *     *           ** **     * **  *  *     *
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AHSV8_DQ868775      GACGACCGAGTTATGGTGAACAAAAGCCAATTGAGGGCACGGATCTTGAAGATTTATAAC
AHSV8_AY163333      GACGATCGAGTTATGGTGAACAAAAGCCAATTGAGGGCACGGATCTTAAAGATTTATAAC
AHSV5_AY163331      AATGATCGCACCTTCGTTAATAAGAGCCAGTTGAAAGTTAGAACTTTGAAGATATATAAT
AHSV6_DQ868774      AGCGGGAAGATGTTAGTTAATAAAAGTAAGTTGAGGGTAAGGAACTTGAAAATTTATAAT
AHSV6_NC_005996     AGCGGAAAGATGTTAGTTAATAAAAGTAAATTAAGGGTAAGGAACTTGAAAATTTACAAT
AHSV9_DQ868776      AGTGACGGCTCAGTGGTGAATAAGAGCAAGCTCCGCGTAAGGGATATGAAGATATATAAT
AHSV4_EU046574      CATGATTCAGGTATAATAAATAAGAGCGTATTGAGAGCGAGAACTCTGAAAATTTACAAT
AHSV4_DQ868773      CATGATTCAGGTATAATAAATAAGAGCGTATTGAGAGCGAGAACTCTGAAAATTTACAAT
AHSV4_D26570        CATGATTCCGGTATAATAAATAAAAGTGTATTGAGAGCGAGAACTCTGAAAATTTACAAT
AHSV1_FJ011108      ACGCGGAAGGCAGTAAAGAATAAGAGTAAACTTGGCGTACGCGATCTGAAAATCTATAAT
AHSV1_AY163329      ACGCGGAAGGCAGTAAAGAATAAGAGTAAACTTGGCGTACGCGATCTGAAAATCTATAAT
AHSV2_AY163332      ACACGTAAAACTGTCAAGAATGGGAGCGCATTGGGAGTCCGAGATTTAAAAATCTACAAT
AHSV1_Z26316        GATAGACATAAGCTGGTTAACAAGAGTGATTTCCGAGTCAGAGAAAATGAAAATATATAAC
AHSV3_U01832        GATAGACATAAGCTGGTTAACAAGAGTGATTTCCGAGTCAGAGAAATGAAAATATATAAC
AHSV3_DQ868772      GATAGACATAAGTTAGTTAATAAGAGTGATTTCCGAGTCAGAGAAATGAAAATATATAAC
AHSV7_AY163330      GATCCGATTTCCGTTGTTAATAAGAGTACTTTTCGGACGAGAGAGATGAAGATTTATAAT
                                                  *    **    **    *       *     * ** ** ** **

AHSV8_DQ868775      AGGGGAAAATTAGATAAATTGATTTTGATTTCAGGGGGGAATTACACTTTCGGGAACAAA
AHSV8_AY163333      AGGGGAATATTAGATAAATTGATTTTGATTTCAGGGGGGAATTACACTTTCGGGAACAAA
AHSV5_AY163331      CGAGGTATGTTGGATAGGTTAATACTAATATCTGGAGGAAATTATACTTTTGGGAATAAG
AHSV6_DQ868774      CGCGGTACGCTTGATTCTTTGTTCTTGATTTCGGGGGGCAGCTATACATTTGGAAATAAG
AHSV6_NC_005996     CGCGGTACGCTTGATTCTTTATTCTTGATTTCGGGTGGCAACTATACATTTGGAAATAAG
AHSV9_DQ868776      AGGTGGGAGGTGGACAGATTAATTTTGATTTCGAGTGGCGATTACACATTTGGGAATAAA
AHSV4_EU046574      AGGGGTTCGATGGATACTTTAATTCTAATTTCGAGTGGAGTTTACACTTTCGGAAATAAA
AHSV4_DQ868773      AGGGGTTCGATGGATACTTTAATTCTAATTTCGAGTGGAGTTTACACTTTCGGAAATAAA
AHSV4_D26570        AGAGGTTCGATGGACACTTTAATTCTGATCTCGAGTGGAGTCTATACCTTTGGAAATAAA
AHSV1_FJ011108      AGAGGGAGAGTTGATAGGTTGATTTTAATTTCGAGCGGTGTTTATACTTTTGGAAATAAG
AHSV1_AY163329      AGAGGGAGAGTTGATAGGTTGATTTTAATTTCGAGCGGTGTTTATACTTTTGGAAATAAG
AHSV2_AY163332      CGAGGGAGAGTAGATAGATTAGTTTTAATTTCGAGCGGCGTTTATACTTTTGGGAACAAA
AHSV1_Z26316        CGAGGACGTTTAGACCGTCTGATATTGATATCTAGTGGTCATTATACATTTGGGAATAAG
AHSV3_U01832        CGAGGACGTTTAGACCGTCTGATATTGATATCTAGTGGTCATTATACATTTGGGAATAAG
AHSV3_DQ868772      CGAGGACGTTTAGACCGTCTGATATTGATATCTAGCGGTCATTATACATTTGGGAATAAG
AHSV7_AY163330      CGAGGAAGGTTAGATAGATTGATTTTGATATCAAGTGGGAATTACACATTCGGTAATAAG
                     *  *     * **     *  *  * ** **  * **    ** ** ** ** ** **

AHSV8_DQ868775      TTCTTGCTCTCAAAACTACTAGCCAAAACAGAGAAG-TAGCGTGACTGCTACTCATGATG
AHSV8_AY163333      TTCTTGCTCTCAAAACTACTAGCCAAAACAGAGAAG-TAGCGTGACTGCTACTCATGATG
AHSV5_AY163331      TTTTTATTATCAAAATTACTCGCCAAGACAGAAAAG-TAGCGTGACTGCTACTCCAGATG
AHSV6_DQ868774      TTCCTATTATCGAAGCTGATGGCAAAAGCCGAATAG-CGGAGTGACTTCCGCTCATGGTG
AHSV6_NC_005996     TTCCTATTATCGAAGCTGATGGCAAAAGCTGAATAG-CGGAGTGACTTCCGCTCATGGTG
AHSV9_DQ868776      TATCTCTTATCGAAACTAATGGCCAAGATCGAACAG-TAGCGTGACTGCTACTCCATGTG
AHSV4_EU046574      TTCTTGTTGTCGAAGTTACTCGCAAAAACGGAATAG-CAACGTGACTGTTGCTCCATGTG
AHSV4_DQ868773      TTCTTGTTGTCGAAGTTACTCGCAAAAACAGAATAG-CAACGTGACTGTTGCTCCATGTG
AHSV4_D26570        TTCTTGTTGTCGAAGTTACTCGCAAAGACGGAATAG-CAACGTGACTGTTGCTCCATGTG
AHSV1_FJ011108      TTTCTCTTCTCAAAGTTATTGTCGAAGATAGAGTAA-CGGTGTGACAACCGTTCCATGCT
AHSV1_AY163329      TTTCTCTTCTCAAAGTTATTGTCGAAGATAGAGTAA-CGGTGTGACAACCGTTCCATGCT
AHSV2_AY163332      TTTTTATTCTCGAAACTACTATCCAAAATAGAGTAA-CGGCGTGACTGCCGTTCTATGCT
AHSV1_Z26316        TTTCTAATGTCTAAGCTGCTTGCGAAAACTGAATAAGCGGAGTGACTCCCGCTCCATGTG
AHSV3_U01832        TTTCTAATGTCTAAGCTGCTTGCGAAAACTGAATAAGCGGAGTGACTCCCGCTCCATGTG
AHSV3_DQ868772      TTTCTGATGTCTAAGCTGCTTGCGAAAACTGAATAAGCGGAGTGACTCCCGCTCCATGTG
AHSV7_AY163330      TTCTTATTATCCAAACTGTTATCCAAGGCAGAGTAAGCGGTGTGACTACCGCTCCAAGTG
                    *   *  * ** **  *  *  * **    **  *     *****     **
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AHSV8_DQ868775       AAT-ACACTTAC-
AHSV8_AY163333       AAT-ACACTTAC-
AHSV5_AY163331       AAT-ACACTTAC-
AHSV6_DQ868774       ATT-CAACTTACC
AHSV6_NC_005996      ATT-CAACTTACC
AHSV9_DQ868776       AAT-ACACTTAC-
AHSV4_EU046574       AAT-ACACATAC-
AHSV4_DQ868773       AAT-ACACATAC-
AHSV4_D26570         AAT-ACACATAC-
AHSV1_FJ011108       GATCACCCTTAC-
AHSV1_AY163329       GATCACCCTTAC-
AHSV2_AY163332       GAATACACATAC-
AHSV1_Z26316         AATCAACT-TAC-
AHSV3_U01832         AATCAACT-TAC-
AHSV3_DQ868772       AATCAACT-TAC-
AHSV7_AY163330       AATCAGCCGTAC-
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Appendix 2

CLUSTAL alignment of full-length AHSV Segment 10

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AHSV4_EU046579      GTTTAAATTATCCCTTGTCATGAATCTAGCTACAATCGCCAAGAATTATAGCATGCATAA
AHSV4_DQ868783      GTTTAAATTATCCCTTGTCATGAATCTAGCTACAATCGCCAAGAATTATAGCATGCATAA
AHSV5_AJ007309      GTTTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAATTATAGCATGCATAA
AHSV6_AHU26171      GTTTAA-TTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAATTATAGTATGCATAA
AHSV9_D12480        GTTTAA-TTATCCCTTGTCATGAATCTAGCTGCAATCGCCGAAAATTATAGTATGCATAA
AHSV9_AJ007308      GTTTAA-TTATCCCTTGTCATGAATCTAGCTGCAATCGCCGAAAATTATAGTATGCATAA
AHSV4_Z48734        GTTTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAATTATAGTATGCATAA
AHSV4_AJ007305      GTTTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAATTATAGTATGCATAA
AHSV4_AHU02712      GTTTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAGAATTATAGTATGCATAA
AHSV6_DQ868784      GTTTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCGAAAATTATAGTATGCATAA
AHSV9_DQ868786      GTTTAAATTATCCCTTGTCATGAATCTAGCTGCAATCGCCAAAAATTATAGCATGCATAA
AHSV8_DQ868785      GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV3_DQ868782      GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV3_NC_006009     GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV3_D12479        GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV3a_AJ007304     GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV3_AJ007303      GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV7_AJ007306      GTTTAAATTATCCCTTGTCATGAGTCTAGCTACGATCGCCGAAAATTATATGATGCATAA
AHSV1_FJ011116      GTTTAA-TTATCCCT-GTCATGAATCTTGCTAGCATCTCCCAAAGCTATATGTCACATAA
AHSV1_U02711        GTTTAAATTATCCCTTGTCATGAATCTTGCTAGCATCTCCCAAAGCTATATGTCACATAA
AHSV8_AJ007307      GTTTAAATTATCCCTTGTCATGAATCTTGCTAGCATCTCCCAAAGCTATATGTCACATAA
AHSV8_U02713        GTTTAAATTATCCCTTGTCATGAATCTTGCTAGCATCTCCCAAAGCTATATGTCACATAA
AHSV2_U59279        GTTTAAATTATCCCTTGTCATGAATCTTGCTAGCATCTCCCAAAGCTATATGTCACATAA
                    ****** ******** ******* *** ***   *** ** * *  ****     *****

AHSV4_EU046579      TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV4_DQ868783      TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV5_AJ007309      TGGAGAGTCGGGGACGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV6_AHU26171      TGGAGAGTCGGAGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV9_D12480        TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV9_AJ007308      TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATTTCGCAAGTGCTCC
AHSV4_Z48734        TGGAGAGTCGGGGGCGATCGTCCCTTATGTGCCACCACCATACAATGTTGCGAGTGCTCC
AHSV4_AJ007305      TGGAGAGTCGGGGGCGATTGTCCCTTATGTGCCACCGCCATATAATTTCGCGAGCGCTCC
AHSV4_AHU02712      TGGAGAGTCGGGGGCGATTGTCCCTTATGTGCCACCGCCATATAATTTCGCGAGCGCTCC
AHSV6_DQ868784      TGGAGAGTCGGGGGCGATTGTCCCTTATGTGCCACCACCATATAATTTCGCGAGCGCTCC
AHSV9_DQ868786      TGGAGAGCAGGGGGCGATCGTCCCATATGTGCCGCCACCATACAATTTCGCAAGTGCTCC
AHSV8_DQ868785      TGGAAATCAGAGGGCAATCGTACCGTATGTTCCGCCCCCTTATACGTATGCAAATGCTCC
AHSV3_DQ868782      TGGAAATCAGAGGGCAATTGTACCGTATGTTCCGCCCCCTTATACGTATGCAAATGCTCC
AHSV3_NC_006009     TGGAAATCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV3_D12479        TGGAAATCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV3a_AJ007304     TGGAAATCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV3_AJ007303      TGGAAATCAGAGAGCAATTGTACCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV7_AJ007306      TGGAAATCAGAGAGCAATTGTGCCGTATGTTCCACCCCCTTATGCGTATGCAAATGCTCC
AHSV1_FJ011116      TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
AHSV1_U02711        TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
AHSV8_AJ007307      TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
AHSV8_U02713        TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
AHSV2_U59279        TGAGAATGAAAGATCAATTGTACCATACATTCCGCCACCGTATCATC---CGACGGCTCC
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AHSV4_EU046579      GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV4_DQ868783      GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV5_AJ007309      GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV6_AHU26171      GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV9_D12480        GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV9_AJ007308      GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV4_Z48734        GACGTTTTCTCAGGGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV4_AJ007305      GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV4_AHU02712      GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV6_DQ868784      GACGTTTTCTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV9_DQ868786      GGCGTTTACTCAGCGTACGAGTCAAATGGAGTCCGTGTCGCTTGGGATACTTAACCAAGC
AHSV8_DQ868785      GACGCTTGGTGGTCAAGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAACCAAGC
AHSV3_DQ868782      GACGCCTGGTGGTCAAGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAACCAAGC
AHSV3_NC_006009     GACGCTTGGTGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAATCAAGC
AHSV3_D12479        GACGCTTGGTGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAATCAAGC
AHSV3a_AJ007304     GACGCTTGGTGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAATCAAGC
AHSV3_AJ007303      GACGCTTGGTGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAATCAAGC
AHSV7_AJ007306      GACGCTTGGTGGTCAGGCGGGTGAAATGGAGTCCATGTCGCTTGGGATACTTAATCAAGC
AHSV1_FJ011116      GGCGCTTGCTGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGATACTTAACCAAGC
AHSV1_U02711        GGCGCTTGCTGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGATACTTAACCAAGC
AHSV8_AJ007307      GGCGCTTGCTGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGATACTTAACCAAGC
AHSV8_U02713        GGCGCTTGCTGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGATACTTAACCAAGC
AHSV2_U59279        GGCGCTTGCTGTATCCGCCAGTCAAATGGAGACCATGTCGCTTGGGATACTTAACCAAGC
                    * **  *  *       *  ** ******** ** ****************** *****

AHSV4_EU046579      CATGTCAAGTACAACTGGTGCGAGTGGGGCGCTTAAAGATGAAAAAGCAGCATTCGGTGC
AHSV4_DQ868783      CATGTCAAGTACAACTGGTGCGAGTGGGGCGCTTAAAGATGAAAAAGCAGCATTCGGTGC
AHSV5_AJ007309      CATGTCAAGTACAACTGGTGCGAGTGGGGCGCTTAAAGATGAAAAAGCAGCGTTCGGTGC
AHSV6_AHU26171      CATGTCAAGTACAACTGGTGCGAGTGGGGCGCTTAAAGATGAAAAAGCAGCATTCGGTGC
AHSV9_D12480        CATGTCAAGTACAACTGGTGCGAGTGGGGCGCTTAAAGATGAAAAAGCGGCATTTGGTGC
AHSV9_AJ007308      CATGTCAAGTACAACTGGTGCGAGTGGGGCGCTTAAAGATGAAAAAGCGGCATTTGGTGC
AHSV4_Z48734        CATGTCAAATACAACTGGTGCGAGTGGGGCGCTTAAAGATGAAAAAGCAGCATTTGGTGC
AHSV4_AJ007305      CATGTCAAGTACAACTGGTGCGAGTGGGGCGCTTAAAGATGAAAAAGCAGCGTTCGGTGC
AHSV4_AHU02712      CATGTCAAGTACAACTGGTGCGAGTGGGGCGCTTAAAGATGAAAAAGCAGCGTTCGGTGC
AHSV6_DQ868784      CATGTCAAGTACAACTGGTGCGAGTGGGGCGCTTAAAGATGAAAAAGCAGCGTTTGGTGC
AHSV9_DQ868786      CATGTCAAGCACAACTGGTGCGAGTGGGGCACTTAAAGATGAAAAAGCAGCATTTGGTGC
AHSV8_DQ868785      CATGTCAAGTACAACTGGTGCGAGTGGGGCCCTTAAGGATGAAAAAGCAGCATTTGGTGC
AHSV3_DQ868782      CATGTCAAGTACAACTGGTGCGAGTGGGGCCCTTAAGGATGAAAAAGCAGCATTTGGTGC
AHSV3_NC_006009     CATGTCAAGTACAACTGGTGCAAGTCGGGCTCTTAAGGATGAAAAAGCAGCGTTTGGTGC
AHSV3_D12479        CATGTCAAGTACAACTGGTGCAAGTCGGGCTCTTAAGGATGAAAAAGCAGCGTTTGGTGC
AHSV3a_AJ007304     CATGTCAAGTACAACTGGTGCAAGTCGGGCTCTTAAGGATGAAAAAGCAGCGTTTGGTGC
AHSV3_AJ007303      CATGTCAAGTACAACTGGTGCAAGTCGGGCTCTTAAGGATGAAAAAGCAGCGTTTGGTGC
AHSV7_AJ007306      CATGTCAAGTACAACTGGTGCAAGTGGGGCTCTTAAGGATGAAAAAGCAGCGTTTGGTGC
AHSV1_FJ011116      AATGTCAAGTTCAGCTGGTGCGAGCGGAGCACTTAAGGATGAAAAGGCAGCGTTTGGAGC
AHSV1_U02711        AATGTCAAGTTCAGCTGGTGCGAGCGGAGCACTTAAGGATGAAAAGGCAGCGTATGGAGC
AHSV8_AJ007307      AATGTCAAGTTCAGCTGGTGCGAGTGGCGCACTTAAGGATGAAAAGGCAGCGTTTGGAGC
AHSV8_U02713        AATGTCAAGTTCAGCTGGTGCGAGTGGCGCACTTAAGGATGAAAAGGCAGCGTTTGGAGC
AHSV2_U59279        AATGTCAAGTTCAGCTGGTGCGAGTGGCGCACTTAAGGACGAAAAGGCAGCGTTTGGAGC
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AHSV4_EU046579      TATGGCGGAAGCATTGCGTGATCCAGAACCCATACGTCAAATTAAAAAGCAGGTGGGTAT
AHSV4_DQ868783      TATGGCGGAAGCATTGCGTGATCCAGAACCCATACGTCAAATTAAAAAGCAGGTGGGTAT
AHSV5_AJ007309      CATGGCGGAAGCATTGCGTGATCCAGAACCCATACGTCAAATTAAAAAGCAGGTGGGTAT
AHSV6_AHU26171      TATGGCGGAAGCATTGCGTGATCCAGAACCCATACGTCAAATTAAAAAGCAGGTGGGTAT
AHSV9_D12480        CATGGCGGAAGCATTGCGTGATCCAGAACCCATACGTCAAATTAAAAAGCAGGTGGGTAT
AHSV9_AJ007308      CATGGCGGAAGCATTGCGTGATCCAGAACCCATACGTCAAATTAAAAAGCAGGTGGGTAT
AHSV4_Z48734        TATGGCGGAAGCATTACGTGATCCAGAACCCATACGTCAGATTAAAAAGCAGGTGGGTAT
AHSV4_AJ007305      TATGGCGGAACGATTGCGTGATCCAGAACCCATACGTCAAATTAAAAAGCAGGTGGGTAT
AHSV4_AHU02712      TATGGCGGAACGATTGCGTGATCCAGAACCCATACGTCAAATTAAAAAGCAGGTGGGTAT
AHSV6_DQ868784      TATGGCGGAAGCATTGCGTGATCCAGAACCCATACGTCAAATTAAAAAGCAGGTGGGTAT
AHSV9_DQ868786      GATGGCGGAAGCATTACGTGATCCAGAACCCATACGTCAAATTAAGAAACAGGTAGGCAT
AHSV8_DQ868785      GATGGCGGAGGCATTACGTGATCCAGAACCAATACGTCAAATTAAGAAAACCGTTGGATT
AHSV3_DQ868782      GATGGCGGAAGCATTACGTGATCCAGAACCAATACGTCAAATTAAGAAAACCGTTGGATT
AHSV3_NC_006009     GATGGCGGAAGCATTACGTGATCCAGAACCGATACGTCAAATAAAGAAACATGTTGGATT
AHSV3_D12479        GATGGCGGAAGCATTACGTGATCCAGAACCGATACGTCAAATAAAGAAACATGTTGGATT
AHSV3a_AJ007304     GATGGCGGAAGCATTACGTGATCCAGAACCGATACGTCAAATAAAGAAACATGTTGGATT
AHSV3_AJ007303      GATGGCGGAAGCATTACGTGATCCAGAACCGATACGTCAAATAAAGAAACATGTTGGATT
AHSV7_AJ007306      GATGGCGGAAGCATTACGTGATCCAGAACCGATACGTCAAATTAAGAAACATGTTGGATT
AHSV1_FJ011116      GGTGGCAGAGGCGTTGAGAGATCCGGAGCCGATCAGAAAAATTAAGCGACAAGTAGGTAT
AHSV1_U02711        GGTGGCAGAGGCGTTGAGAGATCCGGAGCCGATCAGAAAAATTAAGCGACAAGTAGGTAT
AHSV8_AJ007307      GGTGGCGGAGGCGTTGAGAGATCCGGAGCCGATCAGAAAAATTAAGCGACAAGTAGGTAT
AHSV8_U02713        GGTGGCGGAGGCGTTGAGAGATCCGGAGCCGATCAGAAAAATTAAGCGACAAGTAGGTAT
AHSV2_U59279        GATGGCAGAGGCGTTGAGAGATCCGGAGCCGATCAGAAAAATTAAGCGACAAGTAGGTAT
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AHSV4_EU046579      CAGAACTTTAAAGAACCTAAAGATGGAGTTAGCAACAATGCGTCGAAAGAAATCGGCATT
AHSV4_DQ868783      CAGAACTTTAAAGAACCTAAAGATGGAGTTAGCAACAATGCGTCGAAAGAAATCGGCATT
AHSV5_AJ007309      CAGAACTTTAAAGAACCTAAAGATGGAGTTAGCAACAATGCGTCGAAAGAAATCGGCATT
AHSV6_AHU26171      CAGAACTTTAAAGAACCTAAAGATGGAGCTGGCAACAATGCGTCGAAAGAAATCGGCATT
AHSV9_D12480        CAGAACTTTAAAGAACTTAAAGATGGAGTTAGCAACAATGCGTCGAAAGAAATCGGCATT
AHSV9_AJ007308      CAGAACTTTAAAGAACTTAAAGATGGAGTTAGCAACAATGCGTCGAAAGAAATCGGCATT
AHSV4_Z48734        CAGAACTTTAAAAAACTTAAAGATGGAGTTAGTAACAATGCGTCGAAAGAAATCGGCATT
AHSV4_AJ007305      CAGAACTTTAAAGAACTTGAAGATGGAGTTAGCAACAATGCGTCGAAAAAAGTCGGCATT
AHSV4_AHU02712      CAGAACTTTAAAGAACTTGAAGATGGAGTTAGCAACAATGCGTCGAAAAAAGTCGGCATT
AHSV6_DQ868784      CAGAACTTTAAAGAACTTAAAGATGGAGTTAGCGACAATGCGTCGAAAAAAGTCGGCATT
AHSV9_DQ868786      TAGAACCTTGAAAAATTTGAAGACGGAGCTAACGACTATGCGCCGAAAGAAATCGGCATT
AHSV8_DQ868785      AAGGACACTTAAGCATTTAAAGATAGAGTTGGCGTCGATGAGACGTAGGTATGCGATATT
AHSV3_DQ868782      AAGGACGCTTAAGCATTTAAAGATAGAGTTGGCGTCGATGAGACGTAGGTATGCGATATT
AHSV3_NC_006009     AAGAACGCTCAAGCATTTAAAGATAGAGTTGGCGTCAATGAGACGTAGGTATGCGATACT
AHSV3_D12479        AAGAACGCTCAAGCATTTAAAGATAGAGTTGGCGTCAATGAGACGTAGGTATGCGATACT
AHSV3a_AJ007304     AAGAACGCTCAAGCATTTAAAGATAGAGTTGGCGTCAATGAGACGTAGGTATGCGATACT
AHSV3_AJ007303      AAGAACGCTCAAGCATTTAAAGATAGAGTTGGCGTCAATGAGACGTAGGTATGCGATACT
AHSV7_AJ007306      AAGAACGCTCAAGCATTTAAAGATAGAGTTGGCGTCAATGAGACGTAGGTATGCGATACT
AHSV1_FJ011116      CCAAACTCTAAAAACACTGAAAGTTGAATTGAGCGGGATGCGAAGGAAGAAATTGATTTT
AHSV1_U02711        CCAAACTCTAAAAACACTGAAAGTTGAATTGAGCGGGATGCGAAGGAAGAAATTGATTTT
AHSV8_AJ007307      CCAAACTCTAAAAACACTGAAAGTTGAATTGAGCGGGATGCGAAGGAAGAAATTGATTTT
AHSV8_U02713        CCAAACTCTAAAAACACTGAAAGTTGAATTGAGCGGGATGCGAAGGAAGAAATTGATTTT
AHSV2_U59279        CCAAACTCTAAAAGCATTGAAAGTTGAATTGAGCGGGATGCGAAGGAAGAGATTGATTTT
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AHSV4_EU046579      AAAAATAATGATCTTTATTAGTGGATGCGTAACGTTAGCTACATCGATGGTTGGGGGATT
AHSV4_DQ868783      AAAAATAATGATCTTTATTAGTGGATGCGTAACGTTAGCTACATCGATGGTTGGGGGATT
AHSV5_AJ007309      AAAAATAATGATCTTTATTAGTGGATGCGTGACGTTAGCTACATCGATGGTTGGGGGATT
AHSV6_AHU26171      AAAGATAATGATCTTTATTAGTGGATGCGTAACGTTAGCTACATCGATGGTTGGGGGATT
AHSV9_D12480        AAAAATAATGATTTTTATTAGTGGGTGCGTGACGTTAGCTACATCGATGGTTGGGGGATT
AHSV9_AJ007308      AAAAATAATGATTTTTATTAGTGGGTGCGTGACGTTAGCTACATCGATGGTTGGGGGATT
AHSV4_Z48734        AAAAATAATGATTTTCATCAGTGGGTGCGTGACGTTAGCTACATCGATGGTTGGGGGATT
AHSV4_AJ007305      AAAAATAACGATTCTTATCAGCGGATGTGTGACGTTAGCAACATCGATGGTCGGGGGGTT
AHSV4_AHU02712      AAAAATAACGATTCTTATCAGCGGATGTGTGACGTTAGCAACATCGATGGTCGGGGGGTT
AHSV6_DQ868784      AAAAATAATGATTTTTATCAGCGGATGTGTGACGTTAGCAACATCGATGGTCGGAGGGTT
AHSV9_DQ868786      GAAAATAACGATTTTTATCAGTGGGTGTGTGACTTTAGCAACCTCAATGGTTGGAGGATT
AHSV8_DQ868785      ACGAGTAGTCACTTTTATGAGTGGGTGCGTGACAATGGCTACCTCGATGGTGGGCGGGTT
AHSV3_DQ868782      ACGAGTAGTCATTTTTATGAGTGGGTGCGTGACAATGGCTACCTCGATGGTGGGCGGGTT
AHSV3_NC_006009     ACGTGTAGTGATCTTTATGAGCGGGTGCGTAACGATGGCTACCTCGATGGCGGGCGGGTT
AHSV3_D12479        ACGTGTAGTGATCTTTATGAGCGGGTGCGTAACGATGGCTACCTCGATGGCGGGCGGGTT
AHSV3a_AJ007304     ACGTGTAGTGATCTTAATGAGCGGGTGCGTAACGATGGCTACCTCGATGGCGGGCGGGTT
AHSV3_AJ007303      ACGTGTAGTGATCTTAATGAGCGGGTGCGTAACGATGGCTACCTCGATGGCGGGCGGGTT
AHSV7_AJ007306      ACGTGTAGTGATCTTTATGAGCGGGTGCGTAACGATGGCTACCTCGATGGCGGGCGGGTT
AHSV1_FJ011116      GAAAATAATTATGTTTATTTGCGCAAACGTAACTATGGCTACTTCTCTAGTTGGAGGTAT
AHSV1_U02711        GAAAATAATTATGTTTATTTGCGCAAACGTAACTATGGCTACTTCTCTAGTTGGAGGTAT
AHSV8_AJ007307      GAAAATAATTATGTTTATTTGCGCAAACGTAACTATGGCTACTTCTCTAGTTGGAGGTAT
AHSV8_U02713        GAAAATAATTATGTTTATTTGCGCAAACGTAACTATGGCTACTTCTCTAGTTGGAGGTAT
AHSV2_U59279        GAAAATAATTATGTTTATTTGCGCAAACGTAACTATGGCTACTTCTCTAGTTGGAGGTAT
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AHSV4_EU046579      GAGTATCGTTGACGACGAAATATTAAGAGATT---------ATAAGAACAACGATTGGTT
AHSV4_DQ868783      GAGTATCGTTGACGACGAAATATTAAGAGATT---------ATAAGAACAACGATTGGTT
AHSV5_AJ007309      GAGTATCGTTGACGACGAAATATTAGGAGATT---------ATAAGAACAACGATTGGTT
AHSV6_AHU26171      GAGTATCGTTGATGACGAAATATTAAGAGATT---------ATAAGAACAACGATTGGTT
AHSV9_D12480        GAGTATTGTTGATGACCAAATATTAGATGATT---------ATAAGAAAAACGATTGGTT
AHSV9_AJ007308      GAGTATTGTTGATGACCAAATATTAGATGATT---------ATAAGAAAAACGATTGGTT
AHSV4_Z48734        GAGTATTGTTGATGACGATATATTAGAAAATT---------ATAAGACAAATGATTGGTT
AHSV4_AJ007305      AAGTATTGTCGATAACGAAATATTTGAAGATT---------ATAAGAAGAACGATTGGTT
AHSV4_AHU02712      AAGTATTGTCGATAACGAAATATTTGAAGATT---------ATAAGAAGAACGATTGGTT
AHSV6_DQ868784      AAGTATTGTTGATAACGAAATATTTGAAGATT---------ATAAGAAGAACGATTGGTT
AHSV9_DQ868786      AAGCATAGTTGATGACCAGATTTGGGGAGAGT---------ATAAAGATAACGATTGGTT
AHSV8_DQ868785      AACGATTATTGATACAGATATATATAAGGATC---------TTAATAATGATGGATGGCT
AHSV3_DQ868782      AACGATTATTGATACAGAAATATATAGGGATC---------TTAATAGTGATGGATGGCT
AHSV3_NC_006009     AACGATTATTGATAATGAAATATATGAAGACC---------TTAGTGGAGATGGTTGGCT
AHSV3_D12479        AACGATTATTGATAATGAAATATATGAAGACC---------TTAGTGGAGATGGTTGGCT
AHSV3a_AJ007304     AACGATTATTGATAATGAAATATATGAAGACC---------TTAGTGGAGATGGTTGGCT
AHSV3_AJ007303      AACGATTATTGATAATGAAATATATGAAGACC---------TTAGTGGAGATGGTTGGCT
AHSV7_AJ007306      AACGATTATTGATAAAGAAATATATGATGACC---------TTAGTGGAGATGGTTGGCT
AHSV1_FJ011116      GTCAATCGTTGATGAGGATATTGCTAAGCATTTGGCGTTTGACGGAAAAGGGGATTGGGT
AHSV1_U02711        GTCAATCGTTGATGAGGATATTGCTAAGCATTTGGCGTTTGACGGAAAAGGGGATTGGGT
AHSV8_AJ007307      GTCGATCGTTGATGAGGATATTGCTAAGCATTTGGCGTTTGACGGAAAAGGGGATTGGGT
AHSV8_U02713        GTCGATCGTTGATGAGGATATTGCTAAGCATTTGGCGTTTGACGGAAAAGGGGATTGGGT
AHSV2_U59279        ATCAATCGTTGATGAGGATATTGCTAAGCATTTGGCGTTTGACGGAAAGGGAGATTGGGT
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AHSV4_EU046579      AATGAAGACTATACATGGGCTGAATTTGTTATGTACTACAGTTTTGTTAGCGGCGGGTAA
AHSV4_DQ868783      AATGAAGACTATACATGGGCTGAATTTGTTATGTACTACAGTTTTGTTAGCGGCGGGTAA
AHSV5_AJ007309      AATGAAGACTATACATGGGCTGAATTTGTTATGTACTACAGTTTTGTTAGCGGCGGGTAA
AHSV6_AHU26171      AATGAAGACTATACATGGGCTGAATTTGTTGTGTACTACAGTTTTGTTAGCGGCGGGTAA
AHSV9_D12480        GATGAAGACTATACATGGGCTGAATTTGTTATGTACTACAGTTTTGTTAGCTGCGGGTAA
AHSV9_AJ007308      GATGAAGACTATACATGGGCTGAATTTGTTATGTACTACAGTTTTGTTAGCTGCGGGTAA
AHSV4_Z48734        AATGAAAACCATACATGGGCTGAATTTGTTATGTACTACAGTTTTGTTAGCGGCGGGCAA
AHSV4_AJ007305      AATGAAAGCGATACATGGGCTGAATTTGTTATGTACCACAGTTTTGTTGGCGGCGGGTAA
AHSV4_AHU02712      AATGAAAGCGATACATGGGCTGAATTTGTTATGTACCACAGTTTTGTTGGCGGCGGGTAA
AHSV6_DQ868784      AATGAAAACGATACATGGGCTGAATTTGTTATGTACCACAGTTTTGTTGGCGGCAGGTAA
AHSV9_DQ868786      GATAAAAACGATACATGGACTGAACTTATTATGTACAACAGTATTGCTGGCAGCGGGTAA
AHSV8_DQ868785      GTCGAAGACGGTTCACGGTCTGAATCTGCTGTGCACCACCATGTTGTTAGCAGCGGGAAA
AHSV3_DQ868782      GTCGAAGACGGTTCACGGTCTGAATCTGCTGTGCACTACCATGTTGTTAGCAGCGGGAAA
AHSV3_NC_006009     GTCGAAGACGATTCACGGTTTGAATTTGCTGTGTACCACTATGTTGTTAGCGGCTGGAAA
AHSV3_D12479        GTCGAAGACGATTCACGGTTTGAATTTGCTGTGTACCACTATGTTGTTAGCGGCTGGAAA
AHSV3a_AJ007304     GTCGAAGACGATTCACGGTTTGAATTTGCTGTGTACCACTATGTTGTTAGCGGCTGGAAA
AHSV3_AJ007303      GTCGAAGACGATTCACGGTTTGAATTTGCTGTGTACCACTATGTTGTTAGCGGCTGGAAA
AHSV7_AJ007306      GTCGAAGACGATTCACGGTTTGAATCTGCTGTGTACCACTATGTTGTTAGCGGCTGGAAA
AHSV1_FJ011116      GTCAAAAACGGTCCATGGTTTAAATTTATTATGTACCACGATGCTGCTGGCAGCGAATAA
AHSV1_U02711        GTCAAAAACGGTCCATGGTTTAAATTTATTATGTACCACGATGCTGCTGGCAGCGAATAA
AHSV8_AJ007307      GTCAAAAACGGTCCATGGTTTAAATTTGTTATGTACCACAATGCTGCTAGCAGCGAATAA
AHSV8_U02713        GTCAAAAACGGTCCATGGTTTAAATTTGTTATGTACCACAATGCTGCTAGCAGCGAATAA
AHSV2_U59279        GTCAAAAACGGTCCATGGTTTAAATTTGTTATGTACAACGATGCTGCTTGGCAGCGAATAA
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AHSV4_EU046579      GATTTCCGATAAAATGCAAGAGGAGATTTCACGGACTAAACGTGACATTGCGAAAAGAGA
AHSV4_DQ868783      GATTTCCGATAAAATGCAAGAGGAGATTTCACGGACTAAACGTGACATTGCGAAAAGAGA
AHSV5_AJ007309      GATTTCCGATAAAATACAAGAGGAGATTTCACGGACTAAACGTGACATTGCGAAAAGAGA
AHSV6_AHU26171      GATTTCCGATAAAATACAAGAGGAGATTTCACGGACTAAACGTGACATCGCGAAAAGAGA
AHSV9_D12480        GATTTCTGATAAAATACAAGAAGAAATTTCACGGACTAAACGTGACATTGCAAAAAGAGA
AHSV9_AJ007308      GATTTCTGATAAAATACAAGAAGAAATTTCACGGACTAAACGTGACATTGCAAAAAGAGA
AHSV4_Z48734        GATTTCTGATAAAATACAAGAGGAGATTTCACGGACTAAGCGTGACATTGCAAAAAGAGA
AHSV4_AJ007305      GATTTCTGATAAAATACAAGAGGAGATTTCACGAACAAAGCGTGATATTGCGAAAAGAGA
AHSV4_AHU02712      GATTTCTGATAAAATACAAGAGGAGATTTCACGAACAAAGCGTGATATTGCGAAAAGAGA
AHSV6_DQ868784      GATTTCTGATAAAATCCAAGAGGAGATTTCACGAACAAAGCGTGATATTGCGAAAAGAGA
AHSV9_DQ868786      GATTTCCGATAAGATTCAAGAAGAAATTTCGCGGACGAAGCGTGACATTGCGAAGAGAGA
AHSV8_DQ868785      AATATCAGATAAAATACAGGAGGAGATATCACGGACAAAGCGGGATATAGCAAAAAGAGA
AHSV3_DQ868782      AATATCAGATAAAATACAGGAGGAGATATCACGGACAAAGCGGGATATAGCAAAAAGAGA
AHSV3_NC_006009     AATATCAGATAAAATACAGGAGGAGATCTCACGCACAAAGCGGGATATAGCGAAGAGAGA
AHSV3_D12479        AATATCAGATAAAATACAGGAGGAGATCTCACGCACAAAGCGGGATATAGCGAAGAGAGA
AHSV3a_AJ007304     AATATCAGATAAAATACAGGAGGAGATCTCACGCACAAAGCGGGATATAGCGAAGAGAGA
AHSV3_AJ007303      AATATCAGATAAAATACAGGAGGAGATCTCACGCACAAAGCGGGATATAGCGAAGAGAGA
AHSV7_AJ007306      AATATCAGATAAAATACAGGAGGAGATCTCACGTACAAAGCGGGATATAGCGAAGAGAGA
AHSV1_FJ011116      AATATCGGAAAAGGTGAGAGAAGAGATTGCGAGGACAAAAAGAGACATCGCGAAAAGACA
AHSV1_U02711        AATATCGGAAAAGGTGAGAGAAGAGATTGCGAGGACAAAAAGAGACATCGCGAAAAGACA
AHSV8_AJ007307      AATATCGGAAAAAGTGAGAGAAGAGATTGCGAGGACAAAAAGAGACATCGCGAAAAGACA
AHSV8_U02713        AATATCGGAAAAAGTGAGAGAAGAGATTGCGAGGACAAAAAGAGACATCGCGAAAAGACA
AHSV2_U59279        AATATCGGAAAAGGTGAGAGAAGAGATTGCGAGGACAAAAAGAGACATCGCGAAAAGACA
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AHSV4_EU046579      GTCTTACGTGTCAGCGGCGAGTATGTCGTGGAGTGGAGATACTGAGATGTCATTACAGGG
AHSV4_DQ868783      GTCTTACGTGTCAGCGGCGAGTATGTCGTGGAGTGGAGATACTGAGATGTTATTACAGGG
AHSV5_AJ007309      GTCTTACGTGTCAGCGGCGAGTATGTCGTGGAACGGAGATACTGAGATGTCATTACAGGG
AHSV6_AHU26171      GTCTTACGTGTCAGCGGCGAGTATGTCGTGGAATGGAGATACTGAGATGTTATTACAGGG
AHSV9_D12480        GTCTTACGTATCAGCGGCGAGTATGTCGTGGAATGGAGATACTGAGATGTTATTACAGGG
AHSV9_AJ007308      GTCTTACGTATCAGCGGCGAGTATGTCGTGGAATGGAGATACTGAGATGTTATTACAGGG
AHSV4_Z48734        GTCTTACGTATCGGCGGCGAGTATGTCGTGGAGTGGAGATACTGAGATATTATTACAAGG
AHSV4_AJ007305      GTCTTACGTATCTGCGGCGAGTATGTCATGGAATGGAGATACTGAAGTATTATTGCAAGG
AHSV4_AHU02712      GTCTTACGTATCTGCGGCGAGTATGTCATGGAATGGAGATACTGAAGTATTATTGCAAGG
AHSV6_DQ868784      GTCTTATGTATCTGCGGCGAGTATGTCATGGAGTGGAGATACTGAAGTATTATTGCAAGG
AHSV9_DQ868786      GTCTTATGTGTCAGCAGCCAGTATGTCGTGGAGTGGAGATACTGAGATGTTATTACACGG
AHSV8_DQ868785      ATCATACGTTTCTGCGGCTAGTATGTCATGGAGTGGGGATACGAGCGTTTTGTTAAGAGA
AHSV3_DQ868782      ATCATACGTTTCTGCGGCTAGTATGTCATGGAGTGGGGATACGAGCGTTTTGTTAAAAGA
AHSV3_NC_006009     ATCATATGTTTCCGCGGCTAGTATGTCTTGGAGTGGGGATACGAGCGTTCTATTAAAAGA
AHSV3_D12479        ATCATATGTTTCCGCGGCTAGTATGTCTTGGAGTGGGGATACGAGCGTTCTATTAAAAGA
AHSV3a_AJ007304     ATCATATGTTTCCGCGGCTAGTATGTCTTGGAGTGGGGATACGAGCGTTCTATTAAAAGA
AHSV3_AJ007303      ATCATATGTTTCCGCGGCTAGTATGTCTTGGAGTGGGGATACGAGCGTTCTATTAAAAGA
AHSV7_AJ007306      ATCATATGTCCCCGCGGCTAGTATGTCTTGGAGTGGGGATACGAGCGTTCTATTAAAAGA
AHSV1_FJ011116      ATCGTACGTATCAGCTGCGACGATGTCTTGGGATGGCGATAGCGTAACTCTATTACGAGA
AHSV1_U02711        ATCGTACGTATCAGCTGCGACGATGTCTTGGGATGGCGATAGCGTAACTCAATTACGAGA
AHSV8_AJ007307      ATCGTACGTATCAGCTGCGACGATGTCTTGGGATGGCGATAGCGTAACTCTATTACGAGA
AHSV8_U02713        ATCGTACGTATCAGCTGCGACGATGTCTTGGGATGGCGATAGCGTAACTCTATTACGAGA
AHSV2_U59279        ATCGTACGTATCAGCTGCGACGATGTCTTGGGATGGCGATAGCGTAACTCCATTACGAGA
                     ** ** **  * ** ** *  ***** ***   ** ****          **    *

AHSV4_EU046579      AATTAAGTATGGCGAGAGCTAGTATGACCTCCACGAGCGGAAAATCCATCGTGTTGGATG
AHSV4_DQ868783      AATTAAGTATGGCGAGAGCTAGTATGACCTCCACGAGCGGAAAATCCATCGTGTTGGATG
AHSV5_AJ007309      AATTAAGTATGGCGAGAGCTAGTATGACCTCCACAAGCGGAAAATCCATCGTGTTGGATG
AHSV6_AHU26171      AATTAAGTATGGCGAGAGCTAGTATGACCTC-GCGAGCGGAAAATCCATCGTGTTGGATG
AHSV9_D12480        AACTAAGTATGGCGAAAGCTAGTATGACCTCCATGAGCGGAAAATCCATCGTGTTGGATG
AHSV9_AJ007308      AACTAAGTATGGCGAAAGCTAGTATGACCTCCATGAGCGGAAAATCCATCGTGTTGGATG
AHSV4_Z48734        AATTAAGTATGGCGAGAGCTAGTATGACCTCCACGAGCTGAAAATCCATCGTGTTGGATG
AHSV4_AJ007305      AATTAAGTATGGCGATAGCTAGTACGACCTCCACAAGCGGAAAATCCATCGTGTTGGATG
AHSV4_AHU02712      AATTAAGTATGGCGATAGCTAGTACGACCTCCACAAGCGGAAAATCCATCGTGTTGGATG
AHSV6_DQ868784      AATTAAGTATGGCGATAGCTAGTACGACCTCCACAAGCGGAAAATCCATCGTGTTGGATG
AHSV9_DQ868786      AATTAAGTATGGCGAAAGCTAGTATGACCTCCATGAGCGGAAAATCCATCGTGTTGGATG
AHSV8_DQ868785      AGTGAAATATGGCGATAGCTAGAATGACCTCCATTTGTGGGAGATCCACAGTGTTGGGTG
AHSV3_DQ868782      AGTGAAATATGGCGATAGCTAGAATGACCTCCATTTGTGGGAGATCCACAGTGTTGGGTG
AHSV3_NC_006009     GGTAAAATATGGCGACAGCTAGAATGACCTCCATTTGTGGGAGATCCACAGTGTTGGGTG
AHSV3_D12479        GGTAAAATATGGCGACAGCTAGAATGACCTCCATTTGTGGGAGATCCACAGTGTTGGGTG
AHSV3a_AJ007304     GGTAAAATATGGCGACAGCTAGAATGACCTCCATTTGTGGGAGATCCACAGTGTTGGGTG
AHSV3_AJ007303      GGTAAAATATGGCGACAGCTAGAATGACCTCCATTTGTGGGAGATCCACAGTGTTGGGTG
AHSV7_AJ007306      GGTAAATATTGGCGACAGCTAGAATGACCTCCATTAGTGGGAGATCCACAGTGTTGGGTG
AHSV1_FJ011116      TGTAAAATATGGAGACTAGCGGATAGACCTCCAAAAGCGGGATTCCACTGGTGTTGCAGT
AHSV1_U02711        TGTAAAATATGGAGACTAGCGGATAGACCTCCAAAAGCGGGATTCCACTGGTGTTGCAGT
AHSV8_AJ007307      TGTAAAATGTGGAGACTAGCGGATAGACCTCCAAAAGCGGGATTCCGCTGGTGTTGCAGT
AHSV8_U02713        TGTAAAATGTGGAGACTAGCGGATAGACCTCCAAAAGCGGGATTCCGCTGGTGTTGCAGT
AHSV2_U59279        TGTAAAATATGGAGACTAGCGGATAGACCTCCAAAAGCGGGATTCCGCTGGTGTTGCAGT
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AHSV4_EU046579      GATGGAACGCCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV4_DQ868783      GATGGAACGCCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV5_AJ007309      GATGGAACGCCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV6_AHU26171      GATGGAACGCCTAGATCGTCTTCTAGGGAGT-GGGATAACAACTTAC
AHSV9_D12480        GATGGAACGCCTAGATCGTTTTCTAGGGAGT-GGGATAACAACTTAC
AHSV9_AJ007308      GATGGAACGCCTAGATCGTTTTCTAGGGAGT-GGGATAACAACTTAC
AHSV4_Z48734        GATGGAACGCCTAGATCGTTTTCTAGGGAGCCGGGATAACGA-----
AHSV4_AJ007305      GATGGAACGCCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV4_AHU02712      GATGGAACGCCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV6_DQ868784      GATGGAACGCCTAGATCGTCTTCTAGGGAGCCGGGATAACGACTTAC
AHSV9_DQ868786      GATGGAACGCCTAGATCGTTTTCTAGGGAGC-GGGATAACGACTTAC
AHSV8_DQ868785      GATCAAACACCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV3_DQ868782      GATCAAACACCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV3_NC_006009     GATCAAACACCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV3_D12479        GATCAAACACCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV3a_AJ007304     GATCAAACACCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV3_AJ007303      GATCAAACACCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV7_AJ007306      GATCAAACACCTAGATCGTTTTCTAGGGAGCCGGGATAACGACTTAC
AHSV1_FJ011116      GGATAACCGCCTAGATCGTGTTCTAGGGAGCCGGGATAACAACTTAC
AHSV1_U02711        GGATAACCGCCTAGATCGTGTTCTAGGGAGCCGGGATAACGACTTAC
AHSV8_AJ007307      GGATAACCGCCTAGATCGTGTTCTAGGGAGCCGGGATAACGACTTAC
AHSV8_U02713        GGATAACCGCCTAGATCGTGTTCTAGGGAGCCGGGATAACGACTTAC
AHSV2_U59279        GGATAACCGCCTAGATCGTGTTCTAGGGAGCCGGGATAACGACTTAC
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