# Molecular applications of the African horsesickness virus genome segment 2 in diagnostics and epidemiology

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"Twenty years from now you will be more disappointed by the things you didn't do than by the ones you did. So throw off the bowlines, sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream."

**Mark Twain (1835-1910)**

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## Summary

The mortality rate of AHS is more than 90 % in fully susceptible horses. Outbreaks of AHS can spread rapidly and have severe economic consequences. Therefore, the disease is classified in "list A" by the OIE and the international movement of horses is subject to stringent zoosanitary regulations. Apart from prevention by large scale vaccinations, a good knowledge of the epidemiology of the disease together with accurate and rapid diagnostic procedures are the most important means of disease control. The use of genome segment 2 of the virus, in both diagnostic and epidemiological applications, was investigated.

During this study it was shown that cDNA copies of genome segment 2 of the nine reference strain viruses of AHSV hybridize exclusively to viral RNA from their corresponding serotypes. This result provided proof of concept for the feasibility of developing nucleic acid based serotyping procedures. It was followed up by the development of the first two genome segment 2 based serotyping methods. The first is a method that uses a set of nine full-length AHSV genome segment 2 clones as probes to hybridize serotype-specifically to denatured dsRNA. The second method makes use of a universal RT-PCR primer set that was developed for the partial amplification of genome segment 2 of any AHSV serotype. The RT-PCR amplification product is labeled and used as a probe to hybridize with immobilized segment 2 cDNA of the nine serotypes of AHSV in a reverse line blot format. The reverse line blot hybridization method proved to be highly sensitive and accurate and can be used to serotype any AHSV with a single RT-PCR and hybridization within one day. It was further shown that the method is sufficiently sensitive to be performed directly on clinical organ samples.

A set of genome segment 2 clones was also used for the first ever segment 2-based AHSV phylogenetic analysis and molecular epidemiological investigation. Genome segment 2 sequence data was generated from AHSV 7 field cases that occurred in South Africa over a two year period which included an important outbreak in the African horsesickness free area in the Western Cape. The phylogenetic classification was compared with historical and geographical data of the different disease cases. Results

indicated that segment 2 nucleotide sequence data can be used to distinguish between different isolates/strains of one serotype and that the phylogenetic grouping of the isolates reflected their historical and geographical groupings. From this it can be concluded that genome segment 2 sequence data can be used in molecular epidemiological investigations to classify AHSVs according to geographical origin, or topotype. This information can be used to track the movement of a particular virus strain to its origin and implicate possible ways by which the disease is spread from endemic to non-endemic areas.

With the new experimental diagnostic methods that have been developed, the virus can be serotyped directly from clinical organ samples within one day, compared to serological methods which take 2-3 weeks. The phylogenetic analysis demonstrated the advantage of a molecular epidemiological approach to provide definitive data for determining the origin of an AHS outbreak. In future, the use of these new serotyping procedures in conjunction with comprehensive molecular epidemiological data should significantly speed up AHSV diagnostics and contribute to more effective prevention, control and surveillance systems for AHS.

# Opsomming

Perdesiekte is dodelik in meer as 90 % van die gevalle waar vatbare perde geïnfekteer word. Uitbrake van perdesiekte kan baie vinnig versprei en ernstige ekonomiese implikasies tot gevolg hê. Dit het gelei tot die klassifikasie van hierdie siekte in "lys A" deur die OIE en die internasionale vervoer van perde is onderhewig aan streng soösanitêre regulasies. Behalwe vir voorkomende maatreëls wat toegepas kan word deur middel van grootskaalse vaksinerings, is 'n goeie kennis van die epidemiologie van die siekte tesame met akkurate en vinnige diagnostiese metodes, die belangrikste vorms van siektebeheer. Die gebruik van genoomsegment 2 van die virus, in beide diagnostiese en epidemiologiese toepassings, is in hierdie studie ondersoek.

Dit is bewys dat cDNA kopieë van genoomsegment 2 van die nege verwysingstamme van AHSV slegs hibridiseer met virus RNA van ooreenstemmende serotipes. Hierdie resultaat het die vatbaarheid van serotipering deur nukleïensuur gebasseerde metodes bewys. Dit is opgevolg deur die ontwikkeling van die eerste twee genoomsegment 2-gebasseerde serotiperingsmetodes. Die eerste is 'n metode wat 'n stel vollengte perdesiektevirus genoomsegment 2 klone gebruik as peilers vir serotipe-spesifieke hibridisering met gedenatureerde dsRNA. Die tweede metode maak gebruik van 'n stel universele RT-PCR voorvoerders wat ontwikkel is vir die gedeeltelike amplifikasie van genoomsegment 2 van enige perdesiektevirus serotipe. Die RT-PCR produk word gemerk en as 'n peiler gebruik om te hibridiseer met geïmobiliseerde segment 2 cDNA van die nege perdesiekte verwysingstamvirusse in 'n omgekeerde lynklad formaat. Resultate het gewys dat die metode hoogs sensitief en akkuraat is en gebruik kan word om enige perdesiektevirus te serotipeer met 'n enkele RT-PCR en hibridisering, binne een dag. Dit is verder bewys dat die metode sensitief genoeg is dat serotipering direk vanaf kliniese orgaanmonsters gedoen kan word.

'n Stel genoomsegment 2 klone is ook gebruik in die eerste segment 2-gebasseerde filogenetiese analise en molekulêre epidemiologiese ondersoek na die perdesiektevirus. Nukleïensuurvolgorde data is versamel van genoomsegment 2 van AHSV 7-infeksies wat in Suid-Afrika voorgekom het oor 'n tydperk van twee jaar, insluitend 'n belangrike

uitbraak in die perdesiekte-vrye streek in die Wes-Kaap. Die filogenetiese klassifikasie is vergelyk met historiese en geografiese data van die veskillende perdesiekte gevalle. Die resultate het gewys dat genoomsegment 2 nukleïensuurvolgorde data gebruik kan word om te onderskei tussen verskillende isolate/stamme van een serotipe en dat die filogenetiese groepering van die isolate die historiese en geografiese groeperings weerspieël. Hieruit is afgelei dat nukleïensuurvolgorde data van genoomsegment 2 gebruik kan word in molekulêre epidemiologiese ondersoeke om perdesiekte virusse te klassifiseer volgens geografiese oorsprong, of topotipe. Hierdie inligting kan gebruik word om die verspreiding van 'n spesifieke virusstam terug te spoor na sy oorsprong en moontlike maniere aan te dui waardeur die siekte versprei word van endemiese na nie-endemiese gebiede.

Met die nuwe eksperimentele diagnostiese metodes wat ontwikkel is, kan die virus direk vanaf kliniese orgaanmonsters geserotipeer word binne een dag, in teenstelling met serologiese metodes wat 2 - 3 weke duur. Die filogenetiese analise het die voordeel van 'n molekulêre epidemiologiese benadering gedemonstreer om eenduidige data te lewer vir die bepaling van die oorsprong van 'n perdesiekte uitbraak. In die toekoms behoort die gebruik van hierdie nuwe serotiperingsmetodes, tesame met omvattende molekulêre epidemiologiese data, perdesiekte diagnostiek te versnel en by te dra tot die meer effektiewe voorkoming, beheer en monitering van die siekte.

# Abbreviations

| | | |
|---|---|---|
| A | - | adenosine |
| AHS | - | African horsesickness |
| AHSV | - | African horsesickness virus |
| AMV | - | avian myeloblastosis virus |
| bp | - | base pairs |
| BHK | - | baby hamster kidney |
| BSA | - | bovine serum albumin |
| BTV | - | bluetongue virus |
| C | - | cytosine |
| °C | - | degrees Celsius |
| cDNA | - | complementary deoxyribonucleic acid |
| CER | - | chicken embryo-related |
| $cm^2$ | - | square centimeter |
| CPE | - | cytopathic effect |
| DIG | - | digoxigenin |
| DNA | - | deoxyribonucleic acid |
| dCTP | - | deoxycytidine triphosphate |
| $ddH_2O$ | - | double distilled water |
| dNTP | - | deoxynucleotide triphosphate |
| ds | - | double-stranded |
| dTTP | - | deoxythymidine triphosphate |
| dUTP | - | deoxyuracil triphosphate |
| EDTA | - | ethylenediaminetetra-acetic acid |
| EEV | - | equine encephalosis virus |
| EHDV | - | epizootic haemorrhagic disease virus |
| EMEM | - | Eagle's minimum essential medium |
| fg | - | femtogram |
| G | - | guanine |
| g | - | gram, gravitational force |
| GA | - | Gauteng (Province) |

| | | |
|---|---|---|
| $km^2$ | - | square kilometer |
| KZN | - | Kwazulu Natal |
| LiCl | - | lithium chloride |
| M | - | molar |
| MB | - | (suckling) mouse brain |
| mg | - | milligram |
| µl | - | microliter |
| µg | - | microgram |
| ml | - | milliliter |
| mM | - | millimolar |
| MMOH | - | methyl mercuric hydroxide |
| m.o.i. | - | multiplicity of infection |
| ng | - | nanogram |
| nm | - | nanometer |
| NW | - | North West (Province) |
| NS | - | non-structural |
| OIE | - | Office International des Epizooties |
| PCR | - | polymerase chain reaction |
| p.i. | - | post infection |
| pfu | - | plaque forming units |
| RFLP | - | restriction fragment length polymorphism |
| RT | - | reverse transcriptase |
| RNA | - | ribonucleic acid |
| SDS | - | sodium dodecyl sulphate |
| SSC | - | saline-sodium citrate |
| SSPE | - | saline-sodium phosphate-EDTA |
| ss | - | single-stranded |
| STE | - | saline-tris EDTA |
| T | - | thymidine |
| TAE | - | tris-acetate EDTA |
| TE | - | tris-EDTA |
| Tm | - | melting temperature |
| tris | - | tris hydroxymethyl aminoethane |
| UPGMA | - | unweighted pair group method with arithmatic mean |

| | | |
|---|---|---|
| UV | - | ultra violet |
| VN | - | virus neutralization |
| VNT | - | virus neutralization test |
| VP | - | viral protein |
| WC | - | Western Cape (Province) |

# Preface

This thesis is submitted in the form of three research papers and describes the research I carried out under the guidance of Dr. Albie van Dijk at the Onderstepoort Veterinary Institute. Each of the papers include an introduction and discussion and are complete accounts of the studies carried out on different aspects of the use of genome segment 2 of AHSV in diagnostics and molecular epidemiology. Two of the papers have been published and the third will be submitted for publication within the near future. The journals in which the publications have appeared are given at the beginning of the chapters. Author guides can be obtained from:
http://authors.elsevier.com/JournalDetail.html?PubID=506054&Precis=DESC, and
http://authors.elsevier.com/JournalDetail.html?PubID=506080&Precis=DESC.

All the research work was carried out by myself with the following contributions from the co-authors: In Chapters 2 and 4, A.C. Potgieter supplied the genome segment 2 clones of AHSV serotypes 1, 2, 4, 6, 7 and 8 and J.T. Paweska supplied the field and midge pool viruses that were used in the study.

# Contents

# Chapter 1

# General introduction

## 1. Literature review: African horsesickness

### 1.1 Introduction

African horsesickness has had a major impact ever since settlers introduced horses to the continent of Africa. The following appeal appeared in The Veterinary Journal of 1900 under the heading: Horse Sickness at the Front, and gives a good illustration of the feeling of urgency toward the disease: "What we wish to point out is that, as a scourge which annually causes immense loss to the Colonies, it behoves the authorities to take adequate and scientific means to combat it. As yet absolutely nothing is known about its etiology...it is manifest that until the cause is known we cannot hope to battle with the disease. The discovery of the haematozoon of tsetse-fly disease was a notable feather in the cap of the school of research at Cambridge. Cannot some pathologist from the most progressive of Universities help us in like manner with the paarde-ziekte when peace once more reigns from Cape Town to the Zambezi?" (Anon., 1900). It was around this time that dedicated, scientific investigations was initiated in order to identify the causative agent and find a means to prevent the disease.

The first description of the disease using the term "perreziekte" or "pardenziekte" (English horsesickness) appeared during 1719 in official documents of the Cape of Good Hope (Theiler, 1921). Years that were described as being prominent for large outbreaks are 1780, 1801, 1891, 1839 and 1854/1855. The 1854/1855 epizootic was especially bad, causing the loss of 64 850 horses with a value of £ 525 000, in one season. The outbreak was so severe that many landowners gave up horse breeding for sheep farming (Flemming, 1878). This number was noted by Theiler (1921) to constitute about 40% of the total horse population of the Cape Colony at the time. After outbreaks in the Cape colony, the disease was constantly encountered as people trekked

northward to the Transvaal (Henning, 1956) and African horsesickness has been persistently noted in South Africa ever since.

Even after more than 200 years of research, the disease still has devastating effects on the South African horse population and is still regarded as one of the most important veterinary diseases. With increased trade and movement of horses that take part in recreational and sporting events, AHS has become a risk internationally. The translocation of horses from endemic areas is, therefore, strictly controlled in order to avoid the introduction of the disease into non-endemic. The success of these measures rely heavily on the availability of rapid and accurate diagnostic techniques and up to date epidemiological data.

Current routine diagnostic methods, although accurate, depend on virus isolation procedures followed by serological typing of the virus on tissue culture. This takes about two to three weeks. The need has, therefore, been identified to develop new methods that make use of the many advances and contributions that modern molecular techniques have to offer in the fields of surveillance and diagnostics.

In epidemiological studies of infectious diseases the use of molecular sequence data has had a major impact. Fitch and Margoliash (1967) was the first to show that DNA and protein sequence data can be used to clarify the evolutionary history of an organism through the construction and interpretation of phylogenetic trees. With modern automated sequencing methods, sequence data from pathogens is easily obtainable for this application. In addition to this, the availability of high speed computers and free access to software packages like PHYLIP (Felsenstein, PHYLIP ver 3.5c) and MEGA (Kumar et al., 2001), has meant that epidemiological investigations are more frequently carried out with the aid of phylogenetic analysis. To date, the application of molecular sequence data in the study of AHS epidemiology has not been investigated sufficiently. There is currently no clear insight into how molecular sequence data can be used to advance the understanding of AHS epidemiology and its mechanism(s) of spread.

## 1.2 The virus

### 1.2.1 Discovery of the etiological agent.

For a long time there was much confusion about the cause of African horsesickness. One popular conception during the late 19th century was that the disease is caused by some poison or "infective miasma" present in the dew that formed on grass at night. This was concluded after many observations that stabled animals escaped disease, while animals that were left outside and subsequently came into contact with the dew frequently became infected (Flemming, 1878; M'Fadyean, 1900). Until 1887, some investigators also mistakenly suspected anthrax as the cause of the sickness. This was probably due to similar symptoms and the endemic nature of the disease together with some misleading microscopic findings of bacteria in *post mortem* samples. It was soon realized, after more microscopic investigations by veterinary surgeons of the time, J.A. Nunn and D. Hutcheon, that bacteria are not involved (Hutcheon,1881; Hutcheon, 1890; Coley, 1904). It was also noticed from witness accounts that the disease is enzootic in low-lying areas and has a seasonal occurrence, appearing after the first summer rains and disappearing again after the first winter frost. These observations led to the conclusion that the disease has a malarial nature (Coley, 1904; Hutcheon 1890) but the etiological agent remained unknown for the time.

M'Fadayen (1900), and shortly thereafter also Theiler (1901), showed that the causal agent of the disease passed through Berkefeld and Chaimberland filters. These filters were used to remove any bacteria that may have been present in the serum or blood of infected animals. The filtrates were successfully used to infect healthy animals with horsesickness. It was, therefore, concluded that horsesickness was caused by an "ultravisible" organism, from then on referred to as a virus (Theiler, 1903). Theiler further noted that the disease was non-contagious, but that it could easily be inoculated into healthy animals by subcutaneous injection of the blood or serum of sick animals. Attempts to culture the organism outside its natural hosts remained fruitless until Nieschulz (1932) and Alexander (1933) succeeded in serially passaging the virus in mice.

### 1.2.2 Antigenic plurality

Theiler (1903) first observed what is today known as the antigenic plurality or multiple serotypes of the virus when he investigated varying accounts from horse owners about the ability of animals that recovered from horsesickness, to resist getting the disease again. He selected horses that survived an initial challenge and re-infected them. What he observed was that some of the animals resisted the infection completely while others developed clinical signs but recovered and a small number died. He also did challenges with heterologous strain viruses that he obtained from different parts of South Africa. He concluded that although some resistance could be obtained by a horse in this manner, even against a different strain, immunological breakdowns can occur and lead to severe disease or death.

After it became possible to cultivate AHSV in the laboratory, Alexander (1935) developed what he termed a "technique of *in vitro* neutralization". Hyperimmunized horse sera were used to neutralize different viruses to varying extents. This provided a powerful tool for the characterization of antigenic differences between virus strains. There were problems, however, with the use of horse sera. In many cases the horses had antibodies against more than one serotype and this influenced the results. Later on it was also difficult to get enough susceptible horses to type the very large numbers of virus strains that had been collected at the time. This was one of the reasons why McIntosh (1958) decided to make use of hyperimmune rabbit sera. He also believed that since rabbits are not naturally susceptible to the disease, he would only get antibodies to the major antigenic parts of the virus. Through his typing efforts with rabbit sera, he identified 42 different strain of the virus which he could group into seven immunological types. This work was extended by Howell (1962) and he could identify two more serotypes of the virus to distinguish the nine serotypes of AHSV as they are still recognized today.

Immunological protection against AHS is serotype-specific, and for an animal to be fully protected against disease, it has to be immune against all nine serotypes respectively. This impacts both on the development of vaccines and diagnostic procedures. Rapid diagnostic tests are required to identify the virus to the level of serotype for it to be effective in routine application. It is especially in this field of

research where there is scope for applying molecular diagnostic methods which have the big advantage of speed over conventional serological assays.

### 1.2.3 Classification

Initially AHSV was simply classified as an Arbovirus in view of the fact that it was transmitted by blood sucking insects (Ozawa, 1967). It was first noticed that BTV was similar in some respects to reovirus (i.e. resistance of the virion to organic solvents and the dsRNA genome) but that it had distinguishing morphological features (Verwoerd, 1969). The structure of AHSV, in turn, resembled that of BTV closely (Oellermann et al., 1970) and it was shown that the BTV and AHSV genomes are more related to each other than to that of reovirus (Oellermann et al., 1970; Verwoerd and Huismans, 1969). It was then suggested for BTV-like arboviruses to be classified in a group that is separate from the reoviruses and other RNA viruses (Verwoerd, 1969; Murphy et al., 1971) The notion that BTV and AHSV should be classified together in the same genus was confirmed by gel electrophoretic studies of the AHSV and BTV genomes and encoded proteins (Verwoerd et al., 1972; Bremer, 1976). The name *Orbivirus* was proposed for this genus (Borden et al., 1971); "orb" referring to the spherical structures seen in electron micrographs of the viruses.

Today the African horsesickness virus is a member of the family *Reoviridae*. This family contains 9 genera namely: *Orthoreovirus, Orbivirus, Rotavirus, Coltivirus, Aquareovirus, Cypovirus, Fijivirus, Phytoreovirus* and *Oryzavirus*. The *Orbivirus* genus contains 20 confirmed (Table 1) and 13 tentative species or serogroups (Mertens et al., 2000).

Table 1. Confirmed species and number of serotypes in the *Orbivirus* genus

| | |
|---|---|
| *African horse sickness virus* | 9 Serotypes |
| *Bluetongue virus* | 24 Serotypes |
| *Changuinola virus* | 12 Serotypes |
| *Chenuda virus* | 7 Serotypes |
| *Chobar Gorge virus* | 2 Serotypes |
| *Corriparta virus* | 5 Serotypes |
| *Epizootic hemorrhagic disease virus* | 8 Serotypes |
| *Equine encephalosis virus* | 7 Serotypes |
| *Eubenangee virus* | 4 Serotypes |
| *Ieri virus* | 3 Serotypes |
| *Great Island virus* | 36 Serotypes/Isolates |
| *Lebombo virus* | 1 Serotype |
| *Orungo virus* | 4 Serotypes |
| *Palyam virus* | 11 Serotypes |
| *Peruvian horse sickness virus* * | |
| *Umatilla virus* | 4 Serotypes |
| *Wad Medani virus* | 2 Serotypes |
| *Wallal virus* | 3 Serotypes/Strains |
| *Warrego virus* | 3 Serotypes/Strains |
| *Wongorr virus* | 8 Serotypes |

Table from Mertens et al., 2000.

* The Peruvian horse sickness virus species has been added to the *Orbivirus* genus in 2003 (http://www.iah.bbsrc.ac.uk).

### 1.2.4 Genome structure and protein coding

Mirchamsy and Taslimi (1966) showed that the multiplication of AHSV is sensitive to actinomycin D, indicating that it contained an RNA genome. The first virus discovered to have a dsRNA genome, however, was BTV (Verwoerd 1969). Shortly afterwards it was also shown to be true for AHSV (Oellermann et al., 1970). The segmented nature of the genome was demonstrated first with sucrose gradient centrifugation and later with polyacrylamide gel electrophoresis. It was calculated that as for BTV, the genome was made up of 10 segments (Oellerman, 1970; Bremer, 1976) with polyacrylamide gel electrophoretic mobilities of the segments that varied between different serotypes (Bremer et al., 1990).

When purified AHSV proteins were separated by gel electrophoresis, three major and four minor proteins could be identified (Bremer, 1976). The coding assignments of segments 2 and 6 could be deduced from northern blot hybridization patterns of different cloned genome segments (Bremer et al., 1990). The encoded products of the 10 segments were identified by Grubman and Lewis (1992) after comparing *in vitro* translated products from individual genome segments with proteins obtained from infected cells. All together the AHSV genome codes for four major structural proteins (VP2, VP3, VP5 and VP7), three minor structural proteins (VP1, VP4 and VP6) and four non-structural proteins (NS1, NS2, NS3 and NS3a). NS3 and NS3a are encoded from different in-phase initiation codons on segment 10 (Bremer, 1976; Grubman and Lewis, 1992; Van Staden and Huismans, 1991).

### 1.2.5 Structure

The first AHSV structural studies were carried out by Polson (1941). He used ultrafiltration and ultracentrifugation techniques to determine the size of viral particles. The particles were found to have a mean diameter of 50 μm and 45.4 μm respectively using the two methods. He used six different strains of the virus but did not see any significant size differences between them. Electron microscopy was later used by Polson and Deeks (1963), Ozawa et al. (1965) and Oellerman et al. (1970) to characterize the size and morphology of the virus. The size of the virion was measured to be approximately 55 μm and made up of icosahedral capsids constructed of 32 capsomers.

The double-layered structure of the capsid of BTV was described by Verwoerd et al. (1972). Their results showed that the capsid consisted of seven proteins of which two make up a diffuse outer layer that could easily be removed by centrifugation through CsCl gradients at pH 7. Bremer (1976) observed that the two outer proteins could be removed from AHSV particles in the same fashion to reveal an inner capsid made up of five proteins. It was later confirmed that this outer layer of the AHSV particle is made up of VP2 and VP5 and that these two proteins could be selectively released from intact virions (Van Dijk and Huismans, 1982). On this outer layer, VP2 is the protein with the most surface exposure (Lewis and Grubman 1991) and it carries the neutralizing

epitopes of the virus (Burrage et al., 1993). The difference between the serotypes of AHSV is correlated to differences in the genome segments that code for VP2 (Bremer et al., 1990, Potgieter et al., 2003). Work on BTV showed that the minor proteins VP1, VP4 and VP6 are surrounded by VP3, and that VP7 lies on the outside of that (Huismans et al., 1987).

Recently, X-ray crystallography using BTV cores has revealed in more detail the structural arrangements in orbiviruses, and confirmed some of the earlier findings. The three inner core proteins (VP1, VP4 and VP6), together with the 10 dsRNA segments are surrounded by the subcore made up of 120 copies of VP3. Around VP3 are 260 trimers of VP7 and together they constitute the viral core or inner capsid (Grimes et al., 1998; Roy and Sutton et al., 1998; Stuart et al., 1998). From limited structural analysis (Maree et al., 1998) and sequence data, it can be predicted that the structure of AHSV will be very similar.

The arrangement of the BTV dsRNA inside the core has also been investigated using crystallography. It was revealed that the dsRNA is packaged in ordered layers under VP3. These layers of dsRNA are compiled in such a way that they surround putative transcription complexes at the 5-fold axis of symmetry formed by the VP3 molecules (Gouet et al., 1999). This arrangement is presumably necessary for transcription.

## 1.3 The disease

### 1.3.1 Clinical signs and pathology

Four different clinical manifestations of the disease have been described (Theiler, 1921). They are the febrile form (Horsesickness fever), the pulmonary form (Dunkop), the cardiac form (Dikkop) and the mixed form.

The febrile form is a mild manifestation of the disease that frequently remains sub-clinical with the only characteristic an elevated body temperature. It is usually only diagnosed during a *post mortem* examination. Signs of the "dunkop" form include severe dyspnoea, fever and coughing. Fully susceptible animals usually develop this acute form of the disease after a short incubation period of three to five days. In some

cases froth can be seen in the nostrils, but this usually only appears after death. The "dikkop" form is distinguished by subcutaneous swellings to the neck and head. Bulging of the supraorbital fossae and in severe cases, swellings to the eyelids, are typical signs. The disease is sub-clinical and can have an incubation period that varies from five days up to three weeks. The mixed form involves symptoms from both the cardiac and pulmonary forms. These, however, are only identified after death as clinical signs usually manifest as either "dikkop" or "dunkop" or as one followed by the other (Henning, 1956; Coetzer and Erasmus, 1994). Theiler (1903) concluded that the different forms are not due to different viruses. In an experiment he injected two animals under the same conditions and with the same virus after which he observed the "dikkop" form in one and the "dunkop" form in the other.

Very detailed and accurate accounts of the pathology of AHS already appeared in the early scientific literature. These usually consisted of *post mortem* examinations of field cases. A very detailed report was published by Hutcheon (1881) of what he describes as a crude and hurried investigation into the nature of the disease. Nowadays, typical lesions are still described in the same way: There is severe oedema of the lungs that result in filling of the bronchi and trachea with froth that forms as the fluid is mixed with air. The pleural cavity and pericardium are filled with large amounts of fluid (hydrothorax and hydropericardium). Gelatinous oedema are present in the subcutaneous and intermuscular connective tissues, the more acute the case, the more gelatinous the consistency. In some cases the tongue is swollen and blue and often show focal hemorrhaging on the ventral surface. Hemorrhages can also be found on the mucusal surfaces, the intestines and the endocardium (Henning, 1956; Maurer and McCully, 1963; Coetzer and Erasmus 1994).

### 1.3.2 Epidemiology

#### 1.3.2.1 Host species and transmission

Theiler (1903) realized from his own observations and those of others, that AHS is not contagious. With laboratory experiments he demonstrated it to be inocuable by means of subcutaneous injections of blood or serum of an infected into a healthy animal. He concluded that the virus must be contracted through the skin but the way transmission

took place in nature remained a mystery. By looking at similarities between the epidemiology of AHS and malaria, he came to believe that blood-sucking insects are the most likely candidate. This fitted in perfectly with observations that AHS appears in parts of the country that are low-lying and have a moist climate, and that it can spread to high lying areas during rainy seasons. It could also explain why the disease is contracted after sunset when most of these insects are active.

During experiments that were carried out at night over several years, collections of bloodfeeding insects were trapped using horses to attract them. Mistakenly, mosquitoes were suspected to be the vector, but amongst the collections of insects they identified midges of the *Culicoides* genus and included them in experiments to determine if insects that fed on diseased animals, can infect healthy ones (Theiler, 1915). Unfortunately all their efforts were unsuccessful.

Another approach was followed by Du Toit (1944). He demonstrated the presence of the virus in field caught insects by injecting emulsifications he made of the insects into animals, successfully transmitting the disease in this way. He found that collections of midges of the *Culicoides* genus carried both the viruses that cause AHS and BT. Current indications are that *C. imicola* is the principal vector of the disease (Meiswinkel, 1998), but recent reports describe that *C. bolitinos* can act as the main vector in high-lying areas with cooler climates (Meiswinkel et al., 2000; Meiswinkel and Paweska, 2003).

A further question that has to be answered, is what happens to the virus between outbreaks. The only animals, other than horses, that have been shown to die from infection, were dogs (Theiler, 1915). It is known that the blood of all animals that could be infected with the disease and became immune, lost its infectivity. Efforts were, therefore, concentrated on animals that do not show clinical signs. In one search for a possible reservoir, Theiler (1915) gathered as many species as possible around the location of Onderstepoort and collected blood samples which he used for inoculation experiments. Among the candidates were several bird species, wild mammals, reptiles and amphibia, domesticated ruminants, dogs and even humans. He was unsuccessful in all cases. Today there still is no certainty over which vertebrate host(s) act as reservoir(s) for the virus during periods of declined vector activity, but donkeys

(Hamblin et al., 1998) and zebra (Barnard and Paweska, 1993) are indicated as the most likely possibilities based on the fact that they show antibodies against the virus. Direct indication of the virus would be more suitable in determining overwintering hosts. This could be done with conventional virus isolation techniques but this would require a lot of time and effort. A fast molecular technique that indicates the presence of viral RNA would be very beneficial in this regard, making the screening of a large amount of samples less cumbersome. Together with diagnostic applications the development of a rapid nucleic acid based test would be ideally suited for this.

Because the activity and movement of the vector species is sensitive to climatological conditions, the spread of the disease in South Africa is usually seasonal. When periods of drought are succeeded by rainy spells and warmer temperatures, the activity of the vector increases and the occurrence of AHS cases also increase (Meiswinkel et al., 1994). The distance that infected midges travel under normal circumstances is only a few kilometers but it has been suggested that this can be highly increased by wind action (Pedgley and Tucker, 1977; Sellers and Maarouf, 1991). Despite this, the major way that the disease is spread over large distance remains through the transportation of host animals. With air travel these distances are unlimited have been known to span continents (Rodriguez et al., 1992).

### 1.3.2.2 Geographical distribution

AHS was known to be present in Africa as early as 1569 (Theiler, 1921). Ever since then the disease has been considered as enzootic to large areas of sub-Saharan Africa (Henning, 1956). In South Africa only the north-eastern Lowveld area, which includes the Kruger National Park, is thought to be endemic. It is presumed that the disease originates from this area as conditions become favorable for the multiplication and spread of the insect vector (Coetzer and Erasmus, 1994; Bosman et al., 1995). No epidemiological studies have, however, been completed to either prove or disprove these claims. The data that is currently generated from investigations of disease outbreaks can only be used to link viruses to each other based on results from serotyping. From evidence of molecular analysis of BTV genome segments it is known, however, that distinctions can be made within a serotype (De Mattos et al., 1994a; De

Mattos et al., 1994b). Similar investigation of AHSV field isolates could be used to the same effect to provide molecular epidemiological proof for the possibility that the virus may persist in more than one area.

Several historically important epizootics also occurred in non-endemic areas. Among these were several outbreaks in the Middle East. In 1944 the disease appeared in Egypt and Palestine (Alexander, 1948) and during 1959 - 1961 there were large outbreaks that spread very rapidly through the Middle East. Many countries including Afghanistan, Iran, Pakistan, India, Turkey, Syria, Cyprus, Lebanon and Jordan were affected. Towards the end of 1960 the disease was restricted in this area through vigorous vaccination and slaughtering campaigns (Rafyi, 1961). It is estimated that in the region of 300 000 animals including horses and donkeys died during this period (Reid, 1961). Most recently, important outbreaks occurred in Spain (Lubroth 1988, Rodriguez et al., 1992) and later spread to neighboring Portugal in 1989 and eventually even to Marocco in 1989 (Sailleau et al., 1997).

### 1.3.2.3 Molecular epidemiology

Phylogenetic analyses have been applied successfully in epidemiological studies to trace the origins and mechanisms of spread of many human (Scholtissek, 1997; Leitner et al., 1996; Lappalainen et al., 2001) and animal (Greiser-Wilke et al., 2000; Gould et al., 2001; Samuel and Knowles, 2001) viral diseases. In the case of HIV, an infection source has been traced using phylogenetic studies and the results used as legal evidence (Leitner et al., 1996). The relatively high mutation rate that is observed in viruses, make them especially suitable for molecular epidemiological investigations using phylogenetic analysis. Mutations accumulate in viral genomes over time and the resulting genetic differences can be analyzed and used to track the spread of a virus (Vandamme, 2002).

Mutation rates among the RNA viruses are known to be even higher than that of DNA viruses (Drake and Holland, 1999). The RNA polymerase of orbiviruses evidently has no proofreading activity and it has been calculated (Kowalik and Li, 1991) that mutations appear at a rate of $2.2 \times 10^{-3}$ nucleotide substitutions / site / year in BTV

genome segment 1. Although this makes orbiviruses ideal candidates for molecular epidemiological studies through phylogenetic analysis, work has been limited to only a few investigations carried out with BTV. The most important findings showed that sequence comparisons of BTV genome segments 3 (VP3) could be used to separate different isolates into distinct geographical types, or topotypes (Gould, 1987; Gould and Pritchard, 1990; Pritchard et al., 1995). Genome segment 2 (VP2) nucleotide sequences have been used to characterize different lineages in groups of isolates of one serotype (De Mattos et al., 1994a; De Mattos et al., 1994b). Studies have also been carried out comparing sequences from different AHSVs (Zientara et al., 1993; Sailleau et al., 1997; Zientara et al., 1998; Van Niekerk et al., 2001) and although different strains could be identified in some serotypes, no correlation has been shown between their phylogenetic and geographical groupings. One of the aims of this study was, therefore, to gather and investigate the use of sequence data from genome segment 2 for use in molecular epidemiological and topotyping studies.

## 1.4 Control

### 1.4.1 Immunological control and vaccines

One of the first aims of African horsesickness research was to develop a means of protection by preventative inoculation (Theiler, 1903). Early successes were achieved with an immunization technique by which hyperimmune serum was injected together with virus (Theiler, 1907). A total of 3 235 mules were challenged after serum-virus immunization and of these only 66 developed symptoms or died. The same technique was later successfully used to fully protect 85 out of 101 horses (Theiler, 1921).

The next advance in vaccine research came after an attempt was made to find a laboratory substitute for the use of horses as experimental animals. Nieschulz (1932) succeeded in adapting AHSV to grow in mice by serial intracerebral passaging. In so doing, a neurotropic strain of the virus was obtained. Alexander and Du Toit (1934) used neurotropic strains that had been adapted to mouse brains using the same procedure in immunization experiments with horses. Contrary to the results of Nieschulz, they found that these neurotropic viruses were attenuated by serial passage through mice and guinea pigs and could be used to effectively immunize horses. They

even demonstrated immunization against two strains of the virus with a single bivalent injection.

For a long period mouse brain attenuated strains were successfully used as vaccines until reports of post-vaccinal encephalitis were received. This prompted research into the attenuation of the virus by means of tissue culture passage (Erasmus, 1965). The virus was adapted to grow in tissue culture by Erasmus (1963) and Mirchamsy and Taslimi (1963). Subsequently Erasmus (1965) adapted all nine serotypes of AHSV to BHK 21 tissue cultures and used two of these to immunize horses. The animals showed no adverse effects and obtained full immunity against homologous strain challenge. A mixture of tissue culture attenuated, and mouse-brain attenuated viruses grown on tissue culture, have been used as a commercial vaccine until 1990. The formulation was changed after four cases of encephalitis and chorioretinitis occured between 1982 - 1989 in factory workers that came into contact with freeze-dried preparations of this vaccine (Van der Meyden et al., 1992; Swanepoel et al., 1992). The current vaccine is composed of fully tissue culture attenuated strains and is formulated in two separate doses that only contain serotypes 1, 3, and 4 and 2, 6, 7 and 8 respectively (Erasmus, 1978).

Then next major advance in AHS vaccine research came in the form of recombinant subunit vaccines. Roy et al. (1996) and Stone-Marschat et al. (1996) showed that recombinant VP2 could be used on its own to induce protection against lethal challenge. It has since become clear, however, that the adjuvant in the formulation plays an important role in whether the protection from recombinant subunit vaccines is efficient or not (Scanlen et al., 2002).

### 1.4.2 Other methods of control

There is no available therapy for AHS other than the treatment of symptoms and the prevention of secondary infections that may occur (Coetzer and Erasmus, 1994). The only certain means of protecting against the disease is to prevent contact between the animal and the insect vector (Henning, 1956). Even before the vector had been identified, the holding of animals in insect proof stables was prescribed by Theiler

(1921) as a preventative measure. As an alternative he suggested the dressing of the skin with a mixture of paraffin and oil as an insect deterrent. Insecticides are also specified and continuously being investigated for this purpose (Henning, 1956; Coetzer and Erasmus 1994; Braverman and Chizov-Ginzburg, 1998). As a last resort, animals can be moved to areas of higher elevation (Theiler, 1921), a practice that is still recommended by veterinarians during outbreaks in South Africa.

In an attempt to limit the spread of the disease and to prevent outbreaks, international movement control regulations have been implemented. The OIE specifies that horses can only be imported from infected countries after they have been in an AHS-free zone for a period of at least two months and show no signs of the disease at the time of import. If horses are to be imported from infected areas, the animals must be kept in an insect free quarantine facility for a period of 40 days prior to shipment (http://www.oie.int/ eng/normes/mcode/A_00040.htm). The Western Cape Province of South Africa has been identified as an AHS-controlled area to comply with these trade regulations (Bosman en Brückner, 1995). It was regionalized into a protection zone, a surveillance zone and a free zone (Guthrie, 1999) and all horses have to be examined for signs of the disease before they are allowed to be moved into any of these areas from the rest of the country. Long quarantine periods obviously impact negatively on the fitness of animals that are meant to take part in sporting events. This situation can most likely be improved if more sensitive diagnostic techniques are developed for use in the screening of animals to be translocated. Nucleic acid based detection techniques, especially, have the potential of being more sensitive and quicker to perform than the serological techniques that are currently prescribed.

## 1.5 Diagnostics

### 1.5.1 Diagnosis and virus identification

AHS is a notifyable disease and all suspect cases must be reported and investigated. The clinical signs and symptoms that have been described in Section 1.3.1 are usually used in conjunction with factors like the locality, the climatological conditions and the presence of other cases to make a provisional diagnosis. Some of these symptoms and clinical signs, including an elevated body temperature, swellings to the head and

eyelids, respiratory distress and frothy discharges from the nose, can however, be confused with similar symptoms that appear in cases of equine encephalosis. This disease, that also affects horses in South Africa, is caused by the equine encephalosis virus, a virus closely related to AHSV in the *Orbivirus* genus. A laboratory diagnosis is the only sure confirmation of AHS (Henning, 1956; Coetzer and Erasmus, 1994).

Most of the tests that are currently used for orbivirus identification and all the serotyping assays require virus isolation from unclotted whole blood, spleen, lymph node or lung samples. Certain procedures are prescribed by the OIE and are given in the Manual of Standards for Diagnostic Tests and Vaccines. The virus can be isolated either by direct inoculation onto tissue culture, or by two rounds of intracerebral inoculation into two or more newborn mice families. In both cases the presence of virus can be detected by monitoring the pathological effects of the virus. Serotyping assays are then carried out by VNTs. These methods have several distinct disadvantages. They require the use of laboratory animals for virus isolation and the production of antisera to specific serotypes. The virus preparations that are used for production of serological reagents need to be continuously checked for contamination with other viruses. Furthermore, the serotyping procedures themselves take a long time to complete as each virus has to be neutralized with all nine serotype-specific antiserums and the degree of neutralization examined by inoculation of the virus onto tissue culture. Molecular techniques do not suffer from any of these drawbacks. One of the main aims of this study was, therefore, to use the techniques and data that is currently available to develop nucleic acid based methods for the serotype-specific detection of AHSV.

### 1.5.2 Serological tests

Complement fixation (McIntosh, 1956) can be used as a group-specific test to distinguish between AHS and other causes of disease in horses. Equine encephalosis is the only other disease with similar clinical signs, but it rarely results in the death of animals. It is, therefore, common practice in diagnostic facilities to use *post mortem* samples directly for capture-ELISA or virus neutralization tests (personal communication, Dr. Truuske Gerdes, Onderstepoort Veterinary Institute).

Several ELISAs have been developed for the group-specific detection of AHSV. Two sandwich-ELISA techniques that use monoclonal antibodies against VP7 (Laviada et al., 1992) or polyclonal antibodies against whole virus (Hamblin et al., 1991) have been described. These procedures were both found to be sensitive enough to detect virus antigens from infected spleen samples. Anti-AHSV antibodies can be detected in horse sera with an indirect-ELISA that uses whole virus (Hamblin et al., 1990) or recombinant VP7 (Wade-Evans et al., 1993) as antigen. This is currently the method that is prescribed by the OIE for international trade purposes and it is also used for surveillance in the AHS-free zone in the Western Cape.

An indirect-ELISA based on the detection of NS3 has also been described (Laviada et al., 1995). This test can be used to distinguish between naturally infected animals and ones that have been vaccinated with inactivated virus. At the moment inactivated vaccines are not in use, however, which limits the applicability of this test.

### 1.5.3 Nucleic acid based tests

#### 1.5.3.1 Nucleic acid hybridization

Nucleic acid hybridization is increasingly being used as a powerful investigative tool in both veterinary research and routine diagnostics. This is mostly due to the high sensitivity of these techniques and the relative ease with which hybridization probes can be prepared and used. It can be used to either detect a specific target sequence in a sample or to determine the degree of relatedness of two polynucleotide molecules. The latter strategy has been used to initially characterize the relatedness between different orbiviruses (Verwoerd and Huismans, 1969) and different serotypes and genome segments of single species (Huismans and Howel, 1973; Bremer et al., 1990). Hybridization experiments carried out by Bremer et al. (1990) showed that a cloned fragment of genome segment 2 of AHSV 3 only hybridized to RNA from the same serotype. A question that was raised during this study was whether this form of serotype-specific hybridization could be expanded to all the other serotypes and be developed into a nucleic acid based serotyping method for AHSV.

## Hybridization

Probe hybridization is the process where a labeled probe molecule hybridizes with a target molecule to form a hybridization duplex. The hybridization is governed by Watson-Crick base pairing that only allows for distinct pairings between two polynucleotide molecules. It provides for very specific probe and target interactions. Any given 20 nucleotide sequence will only appear once in a random sequence of one trillion nucleotides (Gillespie, 1990). Mismatches or non Watson-Crick base pairings will destabilize a probe-target duplex and the more the mismatches, the more likely it is that the pairing will break up. The stability of a probe-target hybrid is expressed as the melting temperature (Tm). The Tm of a hybridization reaction is defined as the temperature at which 50% of the target molecules in the reaction are hybridized with probe molecules. Apart from nucleotide mismatches, this value can be affected by other factors that include the ionic strength of the solution, GC-content of the probe and target molecules, the presence of chemical destabilizing agents, probe length, and probe concentration (Meinkoth and Whal, 1984).

Probe-target hybridizations can be carried out using several strategies. With *in situ* hybridization (Gall and Pardue, 1969) whole cells or tissues are fixed on a support and the hybridization solution and probe are directly applied to the sample. This technique has the advantage that localized hybridization can be observed in intact tissues or cells. Hybridization can also be carried out between probe and target molecules that are both in solution. The biggest advantage is the speed with which samples can be prepared and used for hybridization. It has the drawback though, that hybridized products have to be purified from unhybridized probe molecules. This purification can be achieved by selectively immobilizing the hybrid, by nuclease digestion of unbound probe or through sandwich hybridization of the hybrid to a second probe that is immobilized on a solid support (Thompson and Gillespie, 1990).

The most widely used technique of immobilizing target nucleic acids onto a solid support, is by blotting it. Nygaard and Hall (1963) and Gillespie and Spiegelman (1965) showed that DNA could be immobilized onto nitrocellulose membranes and hybridized with labeled RNA molecules. DNA could initially not be used as labeled probes as it would bind non-specifically to the nitrocellulose membranes. A method was devised by

Denhardt (1966) to overcome this problem by pre-incubating the membrane with the blotted target in a solution containing bovine serum albumin. Direct blotting of samples is often carried out in a dot blot format (Gillespie and Spiegelman, 1965), immobilizing the target samples with vacuum manifolds of different shapes onto a membrane support. Blotting can also be carried out using nucleic acid samples after they have been fractionated electrophoretically. The fractionated DNA (Southern, 1975) or RNA (Thomas, 1980) samples are blotted by capillary or vacuum action from the gel matrix onto a membrane and used for hybridization. The advantage of using these methods, repectively known as Southern blotting (DNA) and northern blotting (RNA), is that information about the sizes of hybridized targets is available.

With modern equipment it is possible to put a large amount of target dots or lines on small membranes or glass chips. Thousands of different targets are immobilized at known locations on the solid support to form what is referred to as of microarrays. Unfortunately, this technology has limited diagnostic applications to date and is generally used for the study of gene expression profiles (Schena, 1996; Southern et al., 1999).

**Probe preparation**

Apart from probes having to be specific, they must also be very sensitive if they are to be used for diagnostic applications. The higher the probe sensitivity the less of a need exists to amplify the target sequence. A very important factor in determining probe sensitivity, is the method that is chosen for probe labeling and detection. Several enzymatic reactions are available for the efficient labeling of polynucleotides to be used as probes.

A very popular method, known as nick translation (Rigby et al., 1977), involves the repair of nicks created by DNase I in dsDNA molecules with *E. coli* DNA polymerase I. Labeled nucleotides are incorporated into the dsDNA as the polymerase resynthesizes parts of the original molecule. The dsDNA is denatured and the ssDNA molecules are used for hybridization. Probes prepared in this way can be used for very sensitive applications as a high number of labeled nucleotides can be incorporated in the probe.

Feinberg and Vogelstein (1983) described the use of random hexamers to prime DNA synthesis on denatured DNA templates with the Klenow fragment of DNA polymerase I. During this reaction labeled nucleotides are incorporated into the newly synthesized strands. After denaturation, the molecules can be utilized as probes. The advantage of this method is that any template can be used as there is no need for specific primer annealing sequences.

Probes can also be end labeled with T4 polynucleotide kinase, terminal transferase or Klenow fragment. Labeled nucleotides are enzymatically added to either the 5' or 3'-ends of probe molecules (Tabor and Struhl, 1995). Although these methods are easy to use, the number of labeled nucleotides that can be added and therefore, the sensitivity of the probes, is limited.

A highly versatile method of labeling probes is by PCR (Schowalter and Sommer, 1989). Labeled nucleotides are added to the reaction mixture and incorporated in the amplification products. This method has several advantages. 1. The degree of labeling can be altered by varying the concentration of the labeled nucleotides in the reaction. 2. Fragments that are too small to be efficiently labeled by nick-translation or random primer extension reactions, can labeled in this way. 3. Very little starting material is needed as the amount of probe is amplified exponentially by the PCR. This is potentially the biggest advantage when probes are to be used for diagnostic purposes. PCR or RT-PCR is often used to amplify target sequences but the sensitivity of a hybridization assay could also be increased by PCR amplification of the probe. Part of this study was, therefore, focused on the generation of serotype-specific probes by PCR from very little target material and use it in a nucleic acid hybridization assay.

With all the methods described above, labeled dsDNA molecules are produced. These have to be denatured before they can be used as probes. The problem is that the resultant probes all have complementary polynucleotide sequences in the reaction that can re-anneal with each other and not be available for hybridization with the target. This can be avoided by using single-stranded probes. ssDNA probes are synthesized from DNA fragments that are inserted in bacteriophage M13 vectors (Ley et al., 1982). A primer that binds to a specific sequence on the vector initiates the synthesis of a DNA probe that is complementary to the DNA insert. ssRNA probes are made by a

method known as transcript labeling (Melton et al., 1984). The target sequence is cloned into a plasmid containing T7 or SP6 bacteriophage RNA polymerase promoters and transcribed *in vitro*. The drawback of both techniques is that the fragments have to be cloned into vectors with specific primer annealing sequence prior to probe synthesis. Also, before the probes can be used they have to be separated from the vector by gel electrophoresis, or in the case of RNA probes, by DNase treatment.

Probes are labeled with radio-isotopes or with molecules that are chemically attached to the nucleotide and either act as a hapten or catalyze a detectable signal reaction. Radioactive probes have been favored for a long time and the methods described above were all developed using radiolabeled nucleotides. Radiolabeled probes are easily synthesized and are generally more sensitive than non-radioactive probes. $^{32}P$ is the most commonly used isotope for labeling because of its high emission energy and short half life. Nucleotides labeled in this way also do not influence the efficiency of the enzymes used for probe synthesis (Struhl, 1995).

Recently, the use of non-radioactive probes have become more popular. Apart from the obvious advantage of being safer to use and store, these probes are also more stable than radiolabeled probes and can be used long after preparation. Biotin (Langer et al., 1981; Brigati et al., 1983) was the first hapten that was successfully used for the labeling of nucleic acid probes. The biotin label is detected through binding with avidin or streptavidin molecules coupled to alkaline phosphatase that catalyzes a signal reaction. This technique has certain limitations, however, as both the presence of endogenous biotin in biological samples and the tendency of streptavidin to bind non-specifically to nylon membranes can result in high background levels.

The most widely used non-radioactive systems employed today is digoxigenin labeling and anti-DIG-antibody detection (Heiles et al., 1988; Kessler et al., 1989). Using the digoxigenin molecule as a label has several advantages: It does not appear freely in biological samples; high affinity antibodies are available for detection of the label; the label can readily be coupled to nucleotides and is freely available for binding with an anti-DIG antibody; and digoxigenin-labeled nucleotides can be incorporated by DNA and RNA polymerases in labeling reactions (Höltke et al., 1995). The nucleotide that was found to have the best incorporation rate is DIG-dUTP. In labeling reactions it is

used in a 35:65 ratio to dTTP or dUTP. Most enzymatic labeling reactions can be used to incorporate DIG-labeled nucleotides into a probe, but the preferred methods are random primer extension (Höltke et al., 1990) and PCR labeling (Lion and Haas, 1990). Both of these methods can be used to generate hybridization probes with a label density that is required for sensitive applications. PCR labeling has the added benefit that probes can be synthesized from very small amounts of template material.

DIG-labeled probes are detected with sheep anti-digoxigenin antibody Fab fragments that are conjugated with fluorescent dyes or enzymes that catalyze color or chemiluminescent reactions. One of the quickest and most sensitive detection procedures for alkaline phosphatase is with a chemiluminescent reaction (Beck et al., 1989). Subsequently, anti-DIG alkaline phosphatase conjugated antibodies and chemiluminescence has become the most popular procedure for the detection of DIG-labeled probes (Höltke et al., 1992).

### 1.5.3.2 RT-PCR

The direct detection of genomic RNA or DNA molecules for diagnostic purposes is limited by the sensitivity of hybridization assays. The development of PCR (Saiki et al., 1985, 1988; Mullis, 1987) has provided the field of molecular diagnostics with a very powerful tool to overcome this problem. With PCR any DNA fragment can be amplified specifically by making use of heat stable polymerases and short oligonucleotide primers. dsDNA molecules are denatured with heat, after which the primers anneal to the specific target sequences, one on the plus(+) and one on the minus(-) strand to flank the fragment to be amplified. These oligonucleotides then serve as primers for a heat-stable DNA polymerase enzyme which will proceed to synthesize complementary DNA strands using the original DNA strands as templates. The newly formed dsDNA molecules are denatured again and the process is sequentially repeated for a number of cycles, amplifying the DNA exponentially. With modern thermocyclers and optimized reaction conditions, millions of copies of the original target can be amplified after only 20 cycles.

As PCR cannot use RNA as a template, a procedure has been developed that combines reverse transcription of RNA into cDNA, with PCR (Kawasaki et al., 1988). This procedure is known as RT-PCR. Because the RT reaction is also initiated by the annealing of an oligonucleotide primer to a target sequence, RT-PCR can be used to amplify specific fragments with a single primer set.

One of the important applications of RT-PCR has been the diagnostic detection of RNA viruses, and specifically also orbiviruses. Most of these methods are based on the assumption that an amplification product obtained after using a specific primer set during the RT-PCR, indicates the presence of a particular virus. Serogroup-specific RT-PCRs have been developed for BTV (Wade-Evans et al., 1990; Dangler et al., 1990), AHSV (Sakamoto et al., 1994; Mizukoshi et al., 1994; Stone-Marschat et al., 1994; Zientara et al., 1994), EHDV (Wilson, 1994; Harding et al., 1996) and EEV (A.C. Potgieter, unpublished data). This approach has been refined further by using primers that are specific for single serotypes of a virus. RT-PCR primers have been developed that will only amplify specific serotypes of AHSV. The primers are used separately in nine RT-PCRs to determine the virus serotype from purified dsRNA (Sailleau et al., 2000). Although this is a very rapid technique, there are risks involved in relying entirely on the presence of an RT-PCR amplification product to indicate the presence of a particular virus or serotype in a test sample. Sequence variations that can occur in primer binding sites can lead to false negative results. On the other hand the amplification of non-specific products can be incorrectly interpreted as an indication of a specific virus. These risks can only be avoided by verifying the authenticity of the amplification product with a downstream procedure such as restriction enzyme digests (Zientara et al., 1993) or probe hybridizations (Zientara et al., 1998; Akita et al., 1992 ). When such methods are used, the RT-PCR need not be specific to any serotype and can only be employed to indiscriminately amplify target material for the ensuing serotyping step. This particular strategy was investigated in this study in an attempt to increase the sensitivity of serotype-specific hybridization by amplifying a part of genome segment 2 with a universal RT-PCR prior to hybridization.

## 2. Aims of this study

AHS has great national and international importance as a veterinary disease. Consequently, prevention, control and surveillance of AHS require reliable, robust, fast and accurate diagnostic procedures supported by comprehensive epidemiological data. For many medical and veterinary viral pathogens the era of molecular diagnostics and epidemiology is already well established. Research into various aspects of AHS and its etiological agent, AHSV, has been conducted for over a hundred years and excellent advances have been made. Routine diagnostics are, however, still carried out using virological and serological techniques that have been developed 50 years ago and takes 2-3 weeks to complete. Furthermore, epidemiological data is still limited to documenting the serotype of an isolate and information on the location and circumstances of the outbreak.

The lack of cloning procedures for large dsRNA genome segments has been a major constraint in the research and development of recombinant vaccines and new molecular diagnostic procedures for all members of the *Reoviridae* family. The recent development of a successful sequence-independent dsRNA cloning method for complete genome sets (Potgieter et al., 2002) in our laboratory has opened up the field to fully access the power of recombinant DNA technology. This new method was used to generate the first full set of genome segment 2 clones from the nine AHSV serotypes (Potgieter et al., 2003). It is known that the serotypes of AHSV can be distinguished by analyzing differences in the nucleic acid sequence of genome segment 2. Therefore, the main aim of this study was to combine the existing body of molecular knowledge for the *Orbiviruses* and the new set of AHSV genome segment 2 clones to provide proof of concept for the development of new molecular genome segment 2-based serotyping reagents and procedures for AHSV. Two specific aims were formulated:

**A. The primary objective was to demonstrate proof of concept that the complete set of genome segment 2 clones of the nine AHSV serotypes could be developed as serotype-specific probes.**

The following aims were formulated:

1. To determine whether genome segment 2 clones of the nine AHSV serotypes can be used as nucleic acid probes to distinguish between the serotypes.
2. To investigate non-isotopic labeling and detection procedures for the genome segment 2 probes in order to take advantage of the fact that the probes will be safer to use and can be prepared in advance and stored for longer periods.
3. To convert the hybridization procedure to a reverse line blot format to make it possible to perform a single hybridization per serotype and thus speed up the procedure.
4. To investigate the feasibility of increasing the sensitivity of the assay by using probes generated by RT-PCR amplification of a part of genome segment 2.
5. To do a preliminary validation of the reverse line blot assay with different strains and isolates of AHSV.

**B. The second objective was to initiate the first AHSV genome segment 2-based molecular epidemiological dataset.**

The following aims were formulated:

1. To determine if sequence analysis of AHSV genome segment 2 can be used to distinguish between different strains of the virus within a serotype.
2. To ascertain to what extend AHSV genome segment 2 phylogenetic analysis results correlate with the geographical and historical data on the virus isolates.
3. To test the practical use of genome segment 2-based phylogenetic analysis on a set of virus isolates from an AHSV 7 outbreak that occurred in the AHS-free zone in the Western Cape during 1999.

# Chapter 2

## Development of probes for typing African horsesickness virus isolates using a complete set of cloned VP2-genes

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# Development of probes for typing African horsesickness virus isolates using a complete set of cloned VP2-genes

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**Abstract**

A set of cloned full-length VP2-genes from the reference strain of each of the nine serotypes of African horsesickness virus (AHSV) was used to develop probes for typing AHSV isolates. The VP2-gene probes hybridised serotype-specific to purified viral dsRNA from its corresponding serotype. No cross-hybridisation was observed between the different AHSV serotypes or with RNA from equine encephalosis virus or bluetongue virus (BTV) which are related viruses within the genus *Orbivirus* that co-circulate with AHSV in South Africa. The probes were able to detect AHSV isolates from recent field cases of AHS in South Africa, despite being derived from historical reference strains. With regard to sensitivity and time considerations: radioactive $^{32}$P-labelling resulted in a marginal increase in sensitivity over digoxigenin-labelled probes. By infecting cell cultures at different multiplicities of infection (m.o.i.) and harvesting at various times post infection, it was established that AHSV RNA could be detected 16 h post infection (p.i.) at a m.o.i. of 1.00 pfu per cell and 48 h p.i. at a m.o.i. of 0.01 pfu per cell. Typing of AHSV isolates by means of VP2-gene probe hybridisation can be completed in 4 days, which is less than half the time required for conventional isolation and serotyping. This report on the use of a complete set of cloned AHSV VP2-gene probes is the first demonstration of typing for a whole specie (serogroup) in a genus of the family *Reoviridae*. 

*Keywords:* dsRNA viruses; African horsesickness virus; AHSV serotype-specific probes; AHSV typing

## 1. Introduction

African horsesickness (AHS) is an arthropod-borne disease of equines that results in a non-contagious infection which has a mortality rate of more than 90% in susceptible horses. It is an Office International des Epizooties (OIE) list A disease that is endemic in most of sub-Saharan Africa. The disease occurs sporadically in north Africa, Mediterranean countries and the Middle East. The aetiological agent, African horsesickness virus (AHSV) belongs to the genus *Orbivirus* in the family *Reoviridae* (Murphy et al., 1995). There are nine serotypes of AHSV (McIntosh, 1958; Howell, 1962). Since protection to AHS is serotype-specific, horses need to be immune to all

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the nine serotypes in order to be fully protected against disease (Van Dijk, 1999). Outbreaks of AHS outside enzootic regions have major zoosanitary and economic repercussions (Powell, 1985).

For many viruses of medical and veterinary importance, the era of molecular diagnostics is already well established. For viruses for which this technology is not yet in place, molecular methods are increasingly being developed and used for detection, identification and characterisation. Serotyping of AHSVs is important for the rapid implementation of effective vaccination strategies, particularly when outbreaks occur outside enzootic regions, as well as, for proper diagnosis and surveillance of the disease. When the live attenuated polyvalent vaccine produced by Onderstepoort Biological Products (OBP) is to be used in an outbreak, it is important to administer the vaccine that contains the relevant serotype first, since the two AHS vaccine doses have to be administered at least 3 weeks apart (the OBP live attenuated AHS vaccine groups the seven AHSV vaccine strains in two separate bottles, one contains serotypes 1, 3, and 4 and the other serotypes 2, 6, 7 and 8). In specific cases where authorities prefer to administer a monovalent vaccine, such as during the 1987 AHS serotype 4 outbreak in Spain, vaccination can only start once serotyping of isolates is completed. Currently, laboratory confirmation and serotyping of an AHS diagnosis takes about 14 days, since it requires viral isolation and typing. The only methods for serotyping AHSVs to date remains virus neutralisation tests (Howell, 1962; Hopkins et al., 1966; Huismans and Erasmus, 1981).

African horsesickness virions consist of a double-layered capsid comprised of seven structural proteins, VP1–VP7, enclosing a dsRNA genome of ten segments. The outer layer of the capsid is made up of two structural proteins, VP2 and VP5, that surround the inner core particle composed of viral proteins VP1, VP3, VP4, VP6 and VP7 (Bremer, 1976; Van Dijk and Huismans, 1982). The first correlation between the serotype of an orbivirus and the genome date back to cross-hybridisation analyses with bluetongue virus (BTV), the prototype orbivirus, which established that genome segment 2 was the major genome segment that was serotype-specific on hybridisation (Huismans and Howell, 1973). Reassortment studies indicated that the other outer capsid protein, VP5, is also involved in the determination of virus serotype, possibly by imposing conformational constraints on VP2 (Cowley and Gorman, 1989; Mertens et al., 1989). Cross-hybridisation was subsequently used in the characterisation of an Australian BTV isolate (Huismans and Bremer, 1981). An investigation of the antigens involved in serological variation between BTVs demonstrated that BTV VP2 immune precipitated only with the sera obtained from animals infected with homologous serotypes, thus indicating BTV VP2 as the main determinant of serotype-specificity (Huismans and Erasmus, 1981). Similarly, for AHSV, neutralising epitopes were identified on VP2 (Burrage et al., 1993). Subsequently, BTV genome segment 2 was shown to encode VP2 (Van Dijk and Huismans, 1988). Molecular investigations confirmed and extended the data. Gene-assignment studies of cloned fragments from shotgun cloning of BTV dsRNA segments (Huismans and Cloete, 1987) and AHSV3 (Bremer et al., 1990) revealed that VP2-genes hybridised serotype-specific under high stringency conditions. Analyses of nucleic acid sequence data from cloned genes revealed that the VP2-genes of *Orbiviruses* are the most variable genes between serogroups, as well as between serotypes of a serogroup (Iwata et al., 1992; De Mattos et al., 1994a). Investigations on sequence variation between the VP2 of AHSV serotypes are still in their infancy. Thus far, the VP2 genes of only four isolates have been sequenced, namely, one each of AHSV serotypes 3, 4, 6 and 9 which revealed that they have 49% amino acid identity and 67% similarity (Williams et al., 1998).

Group-specific genomic probes of conserved viral genome segments have been developed for several orbivirus serogroups including AHSVs (Bremer et al., 1990), BTVs (Roy et al., 1985; Huismans and Cloete, 1987), equine encephalosis viruses (Viljoen and Huismans, 1989) and epizootic haemorrhagic disease viruses, EHDVs, (Nel and Huismans, 1990). In addition, new serogroup-specific tests such as a capture ELISA (Hamblin et al., 1991) and several RT-PCRs (Stone-Mar-

chat et al., 1994; Zientara et al., 1994, 1995a; Bremer and Viljoen, 1998) have been developed for the AHSV serogroup.

Initial investigations into developing molecular typing methods for at least three *Orbivirus* genera have yielded promising results. In the case of BTV it has been found that VP2 sequences of the BTV isolates are characteristic for each serotype and can assist in typing (Wilson and Chase, 1993). Nucleotide sequence analyses grouped BTV serotypes into nucleotypes in broad agreement with the inter-relationships determined using plaque reduction neutralisation tests (Pritchard and Gould, 1995). For epizootic EHDV1, the concept of VP2 gene-based typing has been demonstrated using an approach combining RT-PCR and probing (Aradaib et al., 1995). The first effort to differentiate AHSV serotypes with a molecular approach was by comparing restriction fragment length polymorphisms (RFLPs) of RT-PCR cDNA prepared from AHSV genome segment 7. A panel of eight restriction enzymes separated the nine AHSV serotypes in two groups based on electrophoretic migration patterns (Zientara et al., 1993). By combining the RFLP data from AHSV genome segment 7 and genome segment 10 cDNA, all the nine AHSV serotypes could be differentiated (Zientara et al., 1995b). However, diagnostic development of this RFLP typing for AHSV was abandoned because neither of the protein products of these segments exerts any detectable influence on virus serotype. Phylogenetic analysis of nucleic acid sequencing data confirmed the relatively low degree of similarity of segment 10 within the AHSV serogroup reflected by RFLP analysis and revealed that the genetic variability between segment 10 of different isolates within a serotype showed extensive divergence compared with the situation amongst BTV serotypes (Sailleau et al., 1997; Zientara et al., 1998).

The aim of this investigation was to extend the observation that a cloned AHSV3 VP2-gene hybridised serotype-specific (Bremer et al., 1990) to a complete set of cloned AHSV VP2-genes and lay the foundation for the diagnostic application of probes for typing AHSV isolates.

Table 1
AHSV reference strains

| Serotype | Year of isolation |
|---|---|
| AHSV 1 | 1962 |
| AHSV 2 | 1961 |
| AHSV 3 | 1963 |
| AHSV 4 | 1962 |
| AHSV 5 | 1962 |
| AHSV 6 | 1963 |
| AHSV 7 | 1962 |
| AHSV 8 | 1962 |
| AHSV 9 | 1961 |

Table 2
1997–1998 field isolates of AHS cases in South Africa

| Isolate | Serotype | Passage history | Source | Year |
|---|---|---|---|---|
| M322 | AHSV3 | 1 MB[a], 2 Vero[b] | Equine spleen | 1997 |
| HS39 | AHSV4 | 2 MB, 1 Vero | Equine blood | 1997 |
| HS6 | AHSV6 | 2 Vero | Equine lung | 1998 |
| HS7 | AHSV8 | 1 MB, 1 Vero | Equine blood | 1998 |
| HS14 | AHSV3 | 1 MB, 2 Vero | Equine spleen | 1998 |
| HS33-4 | AHSV6 | 1 MB, 2 Vero | Equine spleen | 1998 |
| HS37 | AHSV4 | 2 Vero | Equine spleen | 1998 |
| HS43[c] | AHSV7 | 2 Vero | Equine lung | 1998 |
| HS45[c] | AHSV7 | 2 Vero | Equine lung | 1998 |

[a] MB, suckling mouse brain.
[b] Vero, vero cell culture.
[c] Both the HS43 and HS45 isolates were made from Gauteng Province during 1998, but from different horse studs and were not part of the same outbreak.

## 2. Materials and methods

### 2.1. Viruses and cells

AHSV reference strains of serotypes 1–9 (Table 1), AHSV isolates from 1997 to 1998 field cases of AHS in South Africa (Table 2), as well as SA-BTV4 and EEV-Kyalami were obtained from the Onderstepoort OIE Reference Laboratory for Bluetongue and African horsesickness, South

Africa. The passage history of the reference viruses used in this investigation is as follows; virulent isolates from field cases were passaged two to three times in suckling mouse brains, plaque purified twice in Vero cell cultures and freeze-dried. This material was subsequently passaged two to five times in BHK and/or a chicken fibroblast-hamster kidney hybridoma cell line (CER) in modified Eagle's medium supplemented with 10% bovine serum. Viral stocks were titered using Vero cell monolayers as described by Oellermann (1970).

### 2.2. Preparation of DNA probes

Cloned VP2 genes from AHSV3 (Vreede and Huismans, 1994) and AHSV9 (Venter et al., 2000) were obtained from H. Huismans (Department of Genetics, University of Pretoria, South Africa), the cloning of the AHSV5 VP2-gene used was described in Vreede et al. (1998), while full-length cloned VP2-genes of AHSV serotypes 1, 2, 4, 6, 7 and 8 were generated recently (Potgieter et al., manuscript in preparation). Full-length cloned VP2-genes, were excised from plasmids after large scale preparation and isolated by gel electrophoresis. VP2-DNA (0.5 μg) was denatured by boiling and snap cooling on ice. This was used as template to prepare $^{32}$P- or digoxigenin-labelled probes by incorporating either α-$^{32}$P-dCTP (Multiprime DNA labelling system, Amersham Life Sciences) or digoxigenin-11-dUTP (DIG High Prime, Boehringer Mannheim) during random primer extension reactions. After the reactions were completed the probes were denatured by boiling for 5 min.

### 2.3. Preparation of template RNA

Template total viral dsRNA was purified essentially as described by Bremer (1976). Briefly, monolayer cultures of CER cells were infected with virus stocks and harvested at 48–60 h p.i., or earlier when CPE reached 80–100%. The cells were collected by centrifugation, resuspended in STE buffer (0.15 M NaCl, 0.01 M Tris–HCl, pH 7.4, 0.001 M EDTA) and ruptured using a dounce homogeniser. The cells were lysed further with 1% SDS in the presence of Na-acetate buffer (0.01 M Na-acetate pH 5.1, 0.01 M EDTA). The pH of the lysate was adjusted to 5.0 with glacial acetic acid. This was followed by phenol protein extraction and ether extraction to remove residual phenol. RNA was precipitated from the aqueous phase by mixing it with two volumes of ethanol and pelleted by centrifugation at 10 000 × *g* for 45 min. The RNA pellet was dissolved in 0.001 M EDTA and LiCl was added to a final concentration of 2 M to precipitate the ssRNA which was removed by centrifugation (10 000 × *g* for 45 min). The LiCl concentration in the supernatant was adjusted to 4 M to precipitate the dsRNA which was collected by centrifugation as described above, dissolved in RNase-free water and stored at −20°C. The concentration of the dsRNA was determined by UV-spectrophotometry at 260 nm.

In the case of total RNA extraction from viral infected cell culture, the commercial reagent Trizol® (GIBCO-BRL Life Technologies) was used according to the manufacturer's instructions. The method is a modification of the acid guanidinium–phenol extraction procedure described by Chomczynski and Sacchi (1987). Cell cultures were infected as indicated in the text. When CPE reached 80–100% in the cell cultures, total RNA extractions were done. The RNA that was prepared was electrophoresed on a 1% TBE-gel to visually compare the relative amounts of dsRNA between the preparations. The total RNA was denatured with methyl mercury II hydroxide (MMOH) before it was spotted and hybridised with the VP2 gene-probes.

### 2.4. Dot-spot nucleic acid hybridisation

RNA samples were denatured in 20 mM MMOH for 30 min at room temperature and spotted on positively charged nylon membranes (Hybond N$^+$, Amersham) in 200 μl volumes using a 96-well dot-spot apparatus. Unless otherwise indicated, the amount of denatured dsRNA that was applied to the membrane was in the order of 200–600 ng per spot. The membrane was then placed on a filter paper saturated with a solution containing 50 mM NaOH and 1.5 M NaCl for 5 min and neutralised on a filter paper saturated

with a solution containing 1.5 M NaCl, 1 mM EDTA and 0.5 M Tris–HCl, pH 7.2. The RNA was fixed to the membrane by exposure to UV-light (312 nm) for 5 min. Prehybridisation was carried out for 4 h at 42°C in a mixture of 50% formamide, 5 × SSC, 5 × Denhardt's solution (0.02% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin (BSA)) and 0.1% SDS, to which 0.01 mg/ml denatured herring sperm DNA had been added. The membrane was then cut into strips that were hybridised separately with each of the VP2-gene probes as indicated in the text. Generally, as a control, one strip was hybridised with an AHSV group-specific probe, prepared from the AHSV9 NS2-gene (Van Staden et al., 1991). The $^{32}$P-labelled probes were diluted in hybridisation mixture (50% formamide, 5 × SSC, 1 × Denhardt's solution and 0.1% SDS) and hybridisation was carried out at 42°C for 16 h. When digoxigenin-labelled probes were used, both the prehybridisation and hybridisation steps were carried out in DIG Easy Hyb hybridisation solution (Boehringer Mannheim) at 42°C. Where necessary, probes were diluted (in general 10-fold) to avoid non-specific high background hybridisation to the membrane.

### 2.5. Post hybridisation washes and probe detection

Blots were washed twice for 10 min under conditions of high stringency (about 90%), namely in a 0.1 × SSC buffer containing 0.1% SDS, at 65°C to eliminate cross-hybridisation between serotypes of AHSV (Bremer et al., 1990). Radioactive blots were exposed overnight to Hyperfilm MP X-ray film (Amersham Life Sciences) at −70°C with an intensifying screen. Hybridised digoxigenin labelled probes were detected with the use of an anti-DIG-alkaline phosphatase-conjugate antibody and the chemiluminescent substrate, CSPD (DIG High Prime DNA Labelling and Detection Starter Kit II, Boehringer Mannheim). Chemiluminescence was detected on an X-ray film (Hyperfilm MP, Amersham Life Sciences) or with a Lumi-Imager™ F1 workstation.

## 3. Results

### 3.1. Specificity of VP2 gene-probes

The observation that a cloned AHSV3 VP2 gene hybridised serotype-specific to dsRNA from AHSV (Bremer et al., 1990) together with the recent cloning of an AHSV VP2-gene from each of the nine AHSV serotypes (Potgieter et al., manuscript in preparation) offered the first opportunity to investigate the possibility of developing a complete set of probes for typing AHSV isolates. Specificity of the cloned full-length AHSV VP2-gene probes was investigated by hybridising the individual probes to membrane strips containing purified viral dsRNA from the reference strains of AHSV serotypes 1–9 from which the probes were derived, as well as to dsRNA from SA-BTV4 and EEV-Kyalami which are members of two closely related orbivirus species that co-circulate with AHSVs in South Africa. The membranes were washed under conditions that required about 90% stringency, since for BTV the nucleic acid similarity between field isolates, vaccine and reference strains of one serotype is as high as 93.8–99.1% for serotype 17 (De Mattos et al., 1994a), and between 95.2 and 99.9% for serotype 10 (De Mattos et al., 1994b).

The AHSV VP2-gene probes all hybridised to denatured RNA from their corresponding serotypes and no cross-hybridisation was observed between the heterologous AHSV serotypes or with RNA from SA-BTV4 or EEV-Kyalami (Fig. 1). The AHSV serogroup-specific NS2 gene-probe hybridised to RNA from all of the nine AHSV serotypes, but not to RNA from BTV or EEV (Fig. 1). The differences in signal intensities can most likely be ascribed to the variation in amounts of template RNA (200–600 ng) spotted on the membrane referred to in Section 2.

### 3.2. Detection of recent field isolates of AHSV

Since the AHSV VP2-gene probes were all generated from historical AHSV reference strains isolated several decades ago, it was important to determine whether they would detect AHSV isolates from recent AHS field cases in South Africa.

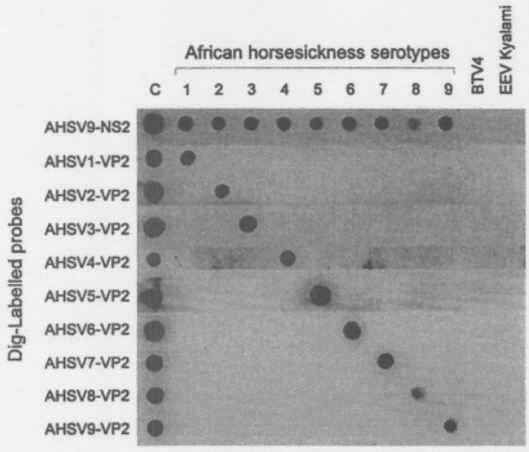


Fig. 1. Chemiluminescent image of a set of membrane strips, each containing denatured dsRNA from the nine serotypes of AHSV and BTV4 and EEV-Kyalami and each hybridised with a different probe as indicated to the left. Probes were prepared by labelling full-length cDNA of the VP2-gene of each of the nine serotypes of AHSV as well as cDNA of the NS2-gene of AHSV9. The NS2-probe was included as an AHSV group-specific control. The positive controls (C) consisted of homologous cloned VP2-DNA.

Two different isolates from the 1997 to 1998 period for each of AHSV serotypes 3, 4, 6 and 7 and one of serotype 8 were available (Table 2). Total RNA was extracted from cell cultures infected with the respective viruses and probed with the different AHSV VP2-gene probes of the serotypes involved.

The AHSV VP2-gene probes were able to detect the recent AHSV field isolates of their corresponding serotypes (Fig. 2). The signals for serotypes 4 and 6 are weaker than the rest, since the probes were used at a 10-fold dilution to reduce background on the nylon membrane.

### 3.3. Sensitivity of VP2-probes

The sensitivity of the type-specific AHSV probes was investigated by two different experimental approaches. The first approach determined the threshold of detection for purified AHSV dsRNA and compared non-radioactive digoxigenin-labelling to $^{32}$P-labelling of the probes under optimised conditions. A dilution series of AHSV7 dsRNA was prepared in RNase-free water and spotted in two rows. The blots were hybridised with a non-radio-active probe and radio-active probe, respectively. The chemiluminescent signal was detected with a Lumi-Imager™ F1 workstation.

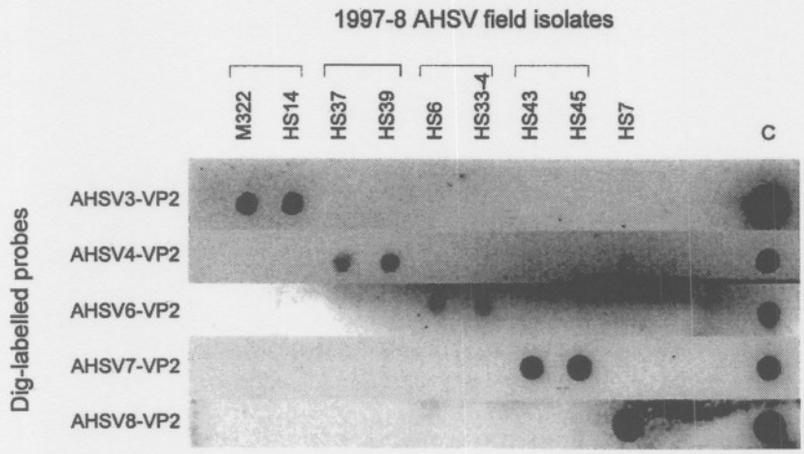


Fig. 2. Chemiluminescent image of VP2-probes obtained from clones of historical AHSV isolates, hybridised with RNA from recent isolates of AHSV (Table 2). Total RNA was extracted from infected cultures and hybridised with each of the VP2-gene probes. The positive controls (C) consisted of homologous cloned VP2-DNA. The serotype from which the digoxigenin-labelled VP2-probes were derived is indicated at the left.

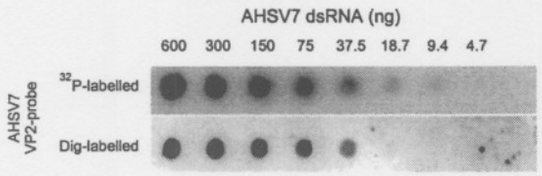


Fig. 3. Autoradiogram and chemiluminescent image of a duplicate dilution series of denatured AHSV7 dsRNA hybridised with $^{32}$P- and digoxigenin-labelled AHSV7 VP2-probes, respectively.

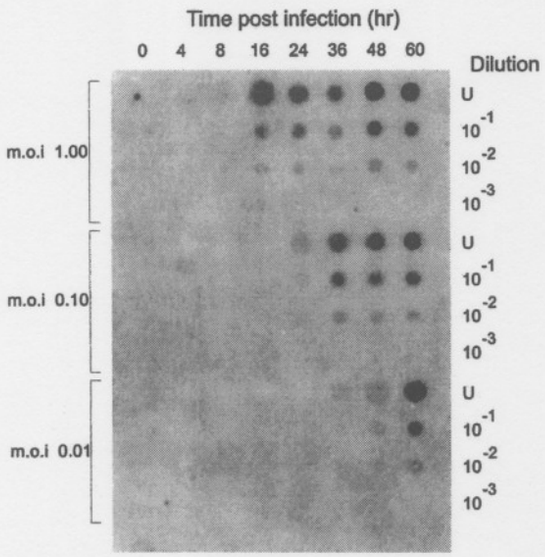


Fig. 4. Autoradiogram of RNA samples extracted from AHSV7 infected cell cultures at different times post infection that were hybridised with an AHSV7 $^{32}$P-labelled VP2-probe. Three sets of infections were done at m.o.i. 1.00, 0.10 and 0.01, respectively. Each RNA sample was denatured and blotted at the different dilutions indicated. The dilution factor of the harvested samples are indicated at the right, U, undiluted.

The digoxigenin-labelled probe could detect 37.5 ng dsRNA, whereas the radio-active $^{32}$P-labelled probe was about four-fold more sensitive, being able to detect in the order of 9.4 ng dsRNA (Fig. 3).

The second approach involved determining the sensitivity of the serotype-specific AHSV probes for viral isolates in cell culture by establishing how soon p.i. at different multiplicities of infection (m.o.i.) there would be enough viral RNA for detection. Three sets of Vero cell cultures were infected using the same AHSV7 inoculum. The tissue cultures were infected at m.o.i. of 1.00, 0.10 and 0.01 plaque forming units (pfu) per cell in 25 cm$^2$ tissue culture flasks. The inoculum was removed after 30 min at 37°C and replaced with fresh medium. At various times p.i. as indicated in Fig. 4, the supernatant was removed from the tissue cultures, discarded and a total RNA extraction was done on the infected cells. At high m.o.i. and at more than 48 h p.i., it was necessary to first collect the cells by low speed centrifugation before the RNA could be extracted from the cells. The RNA was denatured and a 10-fold dilution series of each sample was prepared. The samples were blotted as shown in Fig. 4 and hybridised to the $^{32}$P-labelled AHSV7 VP2-probe.

At m.o.i. of 1.00, viral RNA could be detected 16 h p.i. When the m.o.i. was 0.10, RNA could be detected 24 h p.i. and when the m.o.i. was 0.01, the probe could detect viral RNA from 48 h p.i.

## 4. Discussion

This investigation amounts to proof of concept for the feasibility of genomic typing of AHSV isolates based on a complete set of cloned full-length VP2-gene probes of the nine serotypes of AHSV. It is the first VP2-gene probe procedure to span a whole species (serogroup) in a genus of the family *Reoviridae*. The finding that type-specific AHSV VP2-gene probes derived from historical reference strains detect AHSV isolates from recent field cases of AHS in South Africa and can identify the type in less than half the time needed for current virological procedures, augers well for the future application of the approach as a useful expansion of the diagnostic technology repertoire.

The threshold of detection of radio-active $^{32}$P-labelling of VP2 gene-probes was determined to be about 9 ng total AHSV dsRNA which is in the same range as was reported for the dot and northern blot hybridisation methods for rotavirus detection (Larralde and Flores, 1990; Parwani et al., 1993). $^{32}$P-labelling of probes resulted in a two-fold increase in sensitivity compared to non-radioactive digoxigenin-labelled probes (Fig. 3). When cells were infected with AHSV at a m.o.i of

0.01 pfu per cell, viral RNA could be detected and typed by 48 h p.i. using a $^{32}$P-labelled probe (Fig. 4). The minimum number of cells needed for detection has not been accurately determined, but is estimated to be in the order of $1 \times 10^7$ cells. Cellular RNA does not affect the specificity of the VP2-probes, since no signal was detected in virus-infected cells up to 8 h p.i. (Fig. 4). Further technical refinement can now follow to optimise, evaluate, validate and standardise the procedure for diagnostic applications. Of particular interest for diagnostics will be to cut template RNA extraction and purification requirements to the minimum.

Further improvement of sensitivity of molecular AHSV typing will have to await development of RT-PCR based methods. In the case of rotavirus, a semi-nested RT-PCR method could detect dsRNA from 10 to 10 000 virus particles, which is 200–20 000 times more sensitive than the hybridisation procedure of gene 4 typing (Gentsch et al., 1992; Arista et al., 1999). RT-PCR based typing methods have been initiated for some of the orbiviruses, but none have thus far been developed and tested on all the viral serotypes of a serogroup. For BTV, a multiplex PCR type-specific assay has been developed based on the VP2-gene of the five USA BTV serotypes, generating a specific product which is identified by size difference (Wilson and Chase, 1993). The diagnostic potential of the concept of VP2-based serotyping using a combined RT-PCR/probe approach has also been demonstrated for EHDV1. The sensitivity of the EHDV1 PCR assay was 1.0 fg of virus RNA, the equivalent to 60 virus particles (Aradaib et al., 1995). For AHSV RT-PCR based typing methods to become a reality, a sizeable AHSV VP2-gene sequence reference databank will have to be developed. However, although PCR has numerous advantages for epidemiological studies, production of vaccines, viral evolution investigations, production of non-infectious diagnostic antigens, evaluation of mutagens and can be used for rapid and specific detection and identification of viral RNA, the fact that it can be too specific for some aspects of diagnostics must always be taken into account.

The advantage of orbivirus VP2 gene-probe based typing over RT-PCR-based methods might well prove to be that nucleotide sequence variations which are typical of VP2-genes of BTVs, EHDVs and AHSVs should not affect it in the way it can be expected to impact on RT-PCR based methods where conservation of nucleotide sequences used as primers is crucial. An illustration of the latter problem is the report of a Nigerian rotavirus serotype G8 which could not be typed by PCR due to nucleotide mutation at the 3′ end of the primer binding site (Adah et al., 1997). In order to determine the diagnostic potential of the probe approach, it will thus be necessary to investigate the extent of genetic variability of AHSV VP2-genes within and between serotypes thoroughly. Once this is known, a comprehensive comparison of the merits of molecular and serological serotyping should be undertaken to determine the situation of choice for the application of the various methods.

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# Chapter 3

# One-day AHSV serotyping with a single genome segment 2 RT-PCR amplification and reverse line blot hybridization

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**Abstract**

Rapid serotyping of AHSV is very important for the efficient prevention and control of the spread of AHS outbreaks, especially when they occur in non-endemic regions. This paper describes the first one-day serotyping procedure in which a single RT-PCR is used to amplify the same region of genome segment 2 of all nine AHSV serotypes. It employs a universal primer set of 7 sense and 9 antisense primers which makes it possible to RT-PCR amplify the 5' terminal 521 to 553 bp of genome segment 2 of any AHSV serotype with one reaction. The universal RT-PCR was used to generate serotype-specific digoxigenin labeled probes from dsRNA prepared from infected tissue cultures or organ samples. These probes hybridized serotype-specifically with immobilized cDNA of genome segment 2 of the nine AHSV reference serotypes in a reverse line blot format. The method accurately serotyped all the AHSV reference and vaccine strains as well as a range of field viruses isolated up to 28 years apart. It is also the first molecular method with which it is possible to detect multiple serotypes precisely in co-infected cell cultures in one assay. The sensitivity of the method was sufficient for serotyping AHSV directly from lung and spleen specimens of infected horses.

## 1. Introduction

Fast and accurate serotyping of African horsesickness virus, AHSV (family: *Reoviridae*, genus: *Orbivirus*) is important for the rapid implementation of effective vaccination strategies to control the spread of outbreaks and for up to date epidemiological surveillance of African horsesickness (AHS), one of the most lethal diseases of horses.

AHSV is transmitted by blood feeding *Culicoides* spp. midges (Du Toit, 1944; Meiswinkel and Paweska, 2003). It is an OIE A-listed disease that occurs whenever the insect vector, the virus and the vertebrate host are present in the same region. It is endemic in sub-Saharan Africa and occurs occasionally in north Africa, Mediterranean countries and the Middle East where vector and potential vector midge species are present. Although these and other regions are currently free from the disease, the risk for AHS outbreaks remains high in all areas with a constant presence of the vector species. There are nine AHSV serotypes (McIntosh, 1958; Howell, 1962). Since immunity to AHS is serotype-specific, horses must be immune to all nine serotypes to be fully protected against disease. Commercial live attenuated vaccines have been developed and are produced in South Africa (Van Dijk, 1999). The most effective means of control include the implementation of vaccination campaigns and the enforcement of regulations regarding the movement of equines to and from endemic areas (Bosman et al., 1995). Since vaccination is sometimes used to control the spread of outbreaks, fast and accurate identification of the serotype involved is essential. The only validated method that is currently prescribed for serotyping of AHSV (OIE Manual of Standards for Diagnostic Tests and Vaccines, 2000) consists of virus isolation and identification, followed by virus neutralization tests (VNT) using serotype-specific antisera. This is a laborious process that takes at least two weeks to complete and requires the use of reagents that are prepared in laboratory animals.

The genome of AHSV consist of 10 dsRNA genome segments (Oellermann et al., 1970; Bremer, 1976) that encode seven structural and three non-structural proteins (Bremer et al., 1990; Grubman & Lewis, 1992). Since the advent of recombinant DNA technology, efforts have been made to develop molecular nucleic acid based methods for serotyping AHSV. None have, however, been validated or are widely used. The earliest of these involved the restriction enzyme digestion of genome segment 7 RT-PCR products (Zientara et al., 1993) to differentiate between two groups of serotypes. This was followed by a similar strategy that used RFLPs of genome segment 10 RT-PCR products (Zientara et al., 1995a) that made it possible to identify 8 serotypes. When both tests are used together, all nine serotypes can be distinguished. Currently, there is consensus that genome segment 2 is the ideal target for nucleic acid based serotyping assays. Early hybridization studies reported by Bremer et al. (1990) and subsequent nucleic acid sequencing of cloned full-length genome segments 2 of the nine AHSV serotypes

(Potgieter et al., 2003) established that genome segment 2 has the highest level of nucleic acid variance between serotypes. Genome segment 2 encodes VP2 which is one of two major outer capsid proteins and is the main protective antigen (Scanlen et al., 2002). Serotype-specific genome segment 2 probes have been used to successfully serotype AHSV isolates in 4 days by nucleic acid hybridization (Koekemoer et al., 2000). The first genome segment 2 RT-PCR that has been developed for AHSV serotyping is based on nine primer sets, designed in such a way that each set will only amplify a specific serotype (Sailleau et al., 2000).

The research presented here describes the further development of the concept of genome segment 2 serotype-specific hybridization in a reverse line blot format. It incorporates the sensitivity of RT-PCR into the assay to rapidly and accurately serotype AHSV from isolated virus as well as directly from infected organ samples. This method also has the ability to identify and distinguish between multiple serotypes in the case of mixed infections.

## 2. Materials and Methods

### 2.1 Viruses and cells.

The nine AHSV reference strain viruses (Koekemoer et al., 2000) and the field isolates were obtained from the OIE Reference Center for AHS at the Onderstepoort Veterinary Institute. Vaccine strains were obtained from Onderstepoort Biological Products. Table 1 lists the names and year of isolation of the field viruses that were included in this study. All the virus isolations were performed as follows: Blood samples or homogenates of organs from infected horses were inoculated intracerebrally into newborn mice or diluted with medium and inoculated onto tissue culture. In cases where pathological effects were observed, the virus was passaged on BHK tissue culture and identified by group-specific tests. After another passage, the tissue culture suspensions were freeze-dried in EMEM containing 15% fetal calf serum and 50% buffered lactose peptone, and kept at 4°C. These stocks were reconstituted in EMEM and used to infect BHK cell cultures.

Table 1. AHS field isolate viruses

| Isolate | Serotype | Year of isolation | Isolate | Serotype | Year of isolation |
|---|---|---|---|---|---|
| HS13/99 | 1 | 1999 | HS43/98 | 7 | 1998 |
| HS42/99* | 1 | 1999 | HS45/98* | 7 | 1998 |
| HS11/97 | 2 | 1997 | HS23/98 | 7 | 1998 |
| HS17/98 | 2 | 1998 | MP27/99 | 7 | 1999 |
| HS84/99* | 2 | 1999 | HS82/99 | 7 | 1999 |
| HS14/98 | 3 | 1998 | HS102/99 | 7 | 1999 |
| HS10/03* | 3 | 2003 | HS17/98 | 8 | 1998 |
| HS39/87* | 4 | 1987 | HS2/98 | 8 | 1998 |
| HS3/98 | 4 | 1998 | HS5/98 | 8 | 1998 |
| HS36/98 | 4 | 1998 | HS2/99* | 8 | 1999 |
| HS25/99 | 4 | 1999 | HS4/02 | 8 | 2002 |
| M86/00* | 5 | 2000 | HS2/75* | 9 | 1975 |
| HS66/81 | 6 | 1981 | HS13/01 | 9 | 2001 |
| HS33/98 | 6 | 1998 | HS6/01 | 9 | 2001 |
| HS4/98* | 6 | 1998 | HS15/02 | 9 | 2002 |
| HS23/98 | 7 | 1998 | HS41/02 | 9 | 2002 |

* Isolates used to generate probes for reverse line blot in Fig. 3.

## 2.2 dsRNA isolation

AHSV dsRNA was isolated either from 75cm$^2$ AHSV infected BHK tissue cultures or from infected organ samples. Individual flasks were infected with the different virus strains and kept at 37°C for 2 - 3 days or until clear CPE was observed through 90% of the monolayer. Cells were harvested and collected by low-speed centrifugation and total RNA was extracted using a commercial acid guanidinium-phenol based reagent (TRI-reagent, Molecular Research Center, Inc). The method is based on that of Chomczynski and Sacchi (1987). ssRNA was precipitated from the total RNA solution by adding LiCl to a final concentration of 2 M. Thereafter, the concentration of the LiCl in the solution was increased to 4 M to precipitate the dsRNA. To extract total RNA from organ samples of infected horses, 500 mg quantities of spleen or lung tissue were finely chopped and homogenized with washed sand. The homogenates were mixed with 1 ml TRI-reagent and used for RNA extraction. The precipitated RNA was resuspended in 200 µl double-distilled $H_2O$ and the dsRNA was isolated as described above. The dsRNA was resuspended in 12 µl TE buffer of which 4 µl was used for cDNA synthesis.

## 2.3 Universal RT-PCR

The same method and reagents were used both for the synthesis of cDNA from reference strain virus dsRNA to make the reverse line blots, and to generate digoxigenin-labeled probes from test sample dsRNA. Viral dsRNA was denatured with 20 mM MMOH for 15 min at room temperature after which the MMOH was reduced by the addition of ß-mercapto-ethanol to a final concentration of 20 mM. In both the RT and PCR steps the same universal primer set (Table 2) was used. Reverse transcription was carried out at 42°C for 45 min using 4 µl of the denatured RNA in a 20 µl reaction mixture containing 2 µl 2.5 mM dNTP-mix, 10 pmol of each of the 7 sense and 9 antisense primers, 4 µl cDNA-buffer (50 mM Tris-HCl, pH 8.3; 100 mM KCl; 5 mM $MgCl_2$) and 5 units of AMV reverse transcriptase (Promega). Four microliters of this cDNA-mixture was used for PCR amplification in a total volume of 50 µl. The PCR reaction mixture contained 5 µl 2.5 mM dNTP-mix, 10 pmol of each of the 7 sense and 9 antisense primers, 5 units of Ex-Taq polymerase (Takara) and 5 µl of the supplied 10 × reaction buffer. Thermal cycling conditions were as follows: initial denaturing of the cDNA was carried out for 3

min at 95°C followed by 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 45°C) and extension (1 min at 72°C). The amplicons were separated by electrophoresis on a 1% TAE agarose gel and purified with a gel extraction kit (Qiagen).

## 2.4 Probe preparation

To prepare labeled probes the RT-PCR procedure was followed as described above with the only exception that the dNTP-mixture contained a 1:6 ratio of digoxigenin labeled dUTP (Roche Molecular Biochemicals) to dTTP. Two microliters of the PCR was diluted to 30 µl with water, boiled for 3 minutes and snap cooled in an ice bath and used as a probe for serotype-specific hybridization.

## 2.5 Reverse line blot and hybridization

Line blots were made with denatured genome segment 2-specific cDNA. The same RT-PCR and universal primers described above were used to amplify this cDNA from dsRNA of the nine AHSV reference strain viruses. The amplicons were sequenced to confirm their identity before they were used for making the line blots. To completely denature the cDNA it was boiled for 30 seconds in a buffer containing 0.4 M NaOH and 0.2 M EDTA (pH 8.2) and blotted in 100 ng quantities onto a positively charged nylon membrane (Amersham) in 9 parallel lines using a mini-protean II multiscreen apparatus (Biorad). The cDNA was fixed to the membrane by exposure to UV-light (312 nm) for 5 min. Prehybridization was carried out for 45 min at 42°C in 5 ml DIG Easy Hyb (Roche) hybridization solution.

Hybridization was carried out in the channels of the same apparatus used for making the line blots. The membrane was put into the apparatus with the line blots at 90 degrees to the channels to enable hybridization of all the probes with all the blots in a checkerboard format (Socransky et al., 1994). The wells were filled with 600 µl hybridization solution containing 12 µl denatured probe and allowed to hybridize with the line blots for 3 hours at 42°C. The probe solution was removed from the channels by aspiration and the membrane was removed from the apparatus. The hybridized blots were washed with a 1 x SSPE buffer containing 0.1% SDS, followed by two high stringency washes (0.1 x SSPE, 0.1% SDS) at 68°C. Probe hybridization was detected with a chemiluminescent alkaline

phosphatase detection system and visualized using a Lumi-Imager system (Roche Molecular Systems).

Table 2. Primers used for serotype-independent RT-PCR amplification of genome segment 2 cDNA of the nine AHSV serotypes.

| Sense Primers | | Antisense Primers | | |
|---|---|---|---|---|
| Primer[1] | Sequence[2] | Primer[1] | Sequence[2] | amplicon size |
| UF1 (1,2) | cgcGTTTATTTCAGCATGGC | UR1 | cgCGCTCTCTGTTTATTTCC | 537 |
| UF2 (3,9) | cgcGTTTAATTCACCATGGC | UR2 | cgTCTTACCATCTAAAGAGG | 526 |
| UF3 (4) | cgcGTTAAATTCACTATGGC | UR3 | cgATTTGCGTTTATCGTTCC | 525 |
| UF5 (5) | cgcGTTTATTTCATCATGGC | UR4 | cgATTCTGATCAAGGATTGC | 525 |
| UF6 (6) | cgcGTTTAATTCACCATGGC | UR5 | cgATTCCCGTTAATAGTACC | 525 |
| UF4 (7) | cgcGTTTAATTCACTATGGC | UR6 | cgGCACTCCAACAAATTTCC | 537 |
| UF7 (8) | cgcGTTTAATTCATCATGGC | UR7 | cgACCTTATTTCCATTGACG | 527 |
| | | UR8 | cgCTTTCCATTTGTGTAACG | 553 |
| | | UR9 | cgTCGCATCTCGTACTCCAG | 521 |

[1] The serotype that is amplified by the specific primer is given in brackets after the name of the primer. For the antisense primers the name of the primer corresponds to the serotype.

[2] Nucleotides in capital letters are complementary to the genome segment 2 sequence.

## 3. Results

The aim of this study was to improve the sensitivity and speed of AHSV serotyping based on the genome segment 2 serotype-specific hybridization procedure (Koekemoer et al., 2000). To achieve these goals an RT-PCR was developed for the sensitive and rapid generation of serotype-specific probes from AHSV dsRNA samples with one primer set that is universal for all serotypes. A reverse line blot format was introduced for simultaneous hybridization of the probes to blotted genome segment 2-specific target cDNA of the nine reference strain viruses.

### 3.1 Universal genome segment 2-specific RT-PCR

A set of 16 primers (Table 2) was designed from the nucleic acid sequences of the cloned set of genome segments 2 of the nine AHSV reference strain viruses (Potgieter et al.,

2003). As amplicon size influences the efficiency of RT-PCR, the primers were designed to amplify only the 5'-terminal ~520bp of genome segment 2 of all nine serotypes. The nucleic acid identities between the 5'-ends of serotypes 1 and 2 as well as serotypes 3 and 9, meant that only seven sense primers were needed. The target sites of the antisense primers were selected to keep the sizes of the amplicons within a narrow range to ensure that the RT-PCR is equally efficient for all the serotypes. Furthermore, in order to ensure that all the primers could be used under the same reaction conditions, they were designed to all anneal to the target sequences at the same temperature.

The total of 16 primers (Table 2) were mixed in equimolar amounts and used in a pool for both reverse transcription from the dsRNA and for PCR. Specific products were obtained from all serotypes under identical reaction conditions (Fig. 1a). When probes were prepared, the amplicons showed a molecular weight increase that was due to the presence of the digoxigenin hapten molecules. The difference between a labeled and unlabelled product is visible on the agarose gel in Fig. 1b.

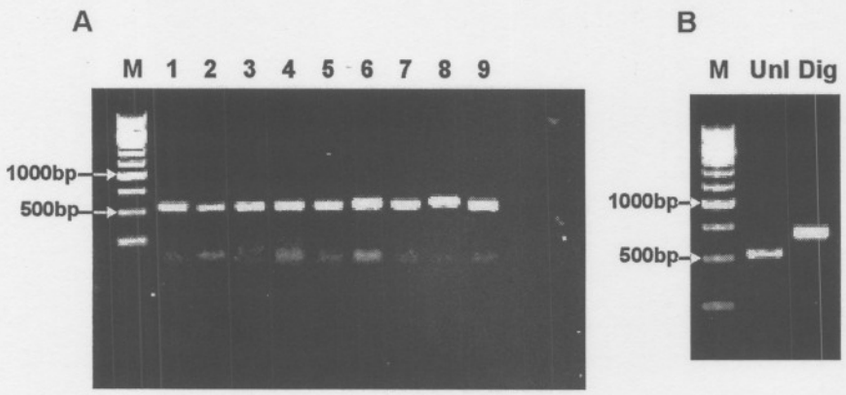


**Fig. 1.** (A) RT-PCR amplification products obtained from the dsRNA of the nine serotypes of AHSV. All the reactions were carried out using one universal primer set and identical reaction conditions. This reaction was used to generate probes from virus dsRNA by incorporating a digoxigenin label into the PCR product. (B) The presence of digoxigenin-labeled nucleotides in the probe (Dig), increased its molecular weight as compared to that of an unlabeled (Unl) RT-PCR product.

### 3.2 Serotype-specific hybridization with reference and vaccine strains

To speed up and simplify the procedure of serotyping by hybridization the protocol was changed from the traditional published format of probing each target with nine probes (Koekemoer et al., 2000), to a reverse line blot format in which the 9 serotype-specific targets can be hybridized simultaneously. Probes were generated by RT-PCR amplification from dsRNA test samples, and hybridized to denatured genome segment 2 cDNA of the nine AHSV reference strains that were blotted in 9 parallel lines on a membrane. The serotype of the virus could then be determined from the position where the probe hybridized. The line blots were hybridized with probes prepared from different dsRNA samples, washed under high stringency conditions and the probe signals were detected by chemiluminescence (Fig. 2). This procedure correctly serotyped all nine the AHSV reference strain (Fig. 2a) and the seven vaccine strain viruses (Fig. 2b). In all cases hybridization was serotype-specific and there was no cross-hybridization when using either the vaccine or reference strain dsRNA as templates for probe preparation.

Another property of the universal primer set is that more than one probe can be prepared with one reaction from a sample. If the sample contains viruses from different serotypes, it will become apparent after hybridization. The AHS vaccine is used in South Africa as two separate multivalent preparations. The first contains serotypes 1, 3, 4 and the second serotypes 2, 6, 7 and 8. To test whether the RT-PCR reverse line blot procedure can detect multiple AHSV serotypes, two tissue culture flasks were respectively co-infected with the two groups of viruses as they are present in the vaccine preparations. Probes generated from dsRNA extracted from the co-infected cell cultures accurately identified the serotypes of all the viruses in the mixed infected cultures in a single assay (Rows I and II, Fig. 2b). Weaker signals were a result of the viruses not growing to the same titers that are observed when cells were infected with a single virus.

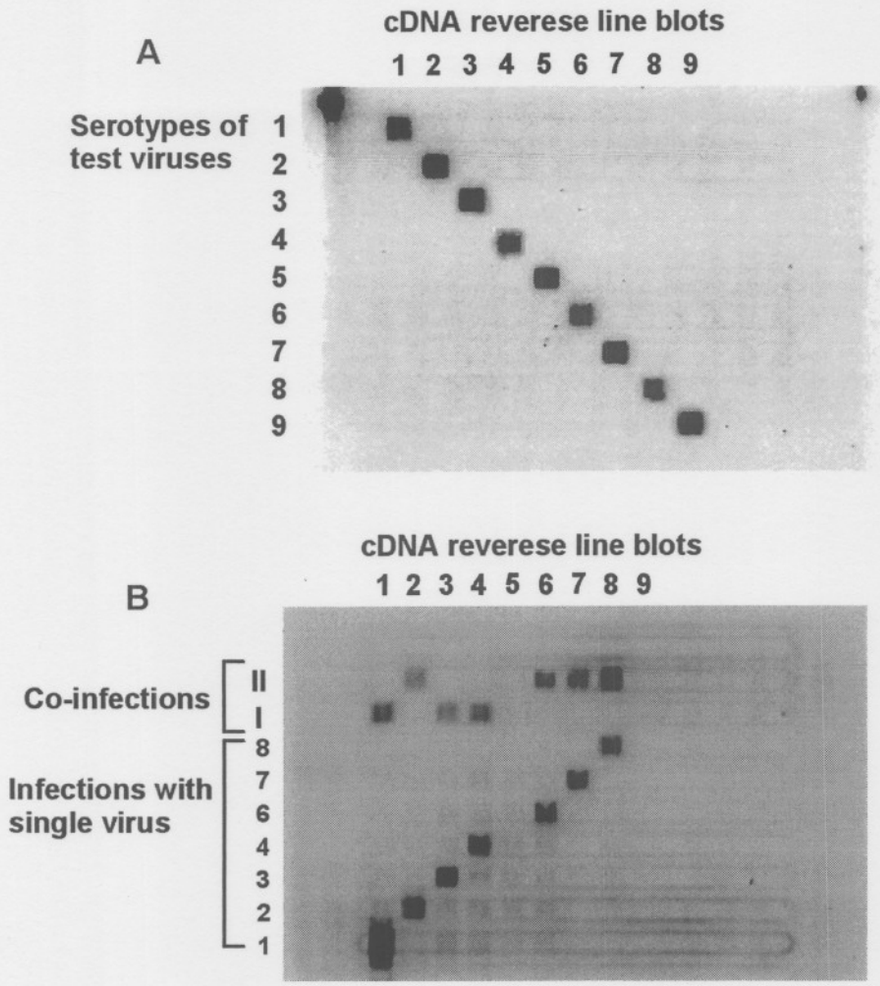


**Fig. 2.** Reverse line blot hybridizations after stringency washes and chemiluminescent detection. The reverse line blots were made by immobilizing segment 2-specific cDNA in 9 parallel lines on the membrane. The line blots run vertically on the figures. (A) Blots were hybridized with probes that were prepared from dsRNA of the nine reference strains of AHSV. (B) Serotype-specific hybridizations were carried out with probes prepared from the dsRNA of the seven vaccine strain viruses. The serotypes of the vaccine viruses are indicated at the left. The top part of the blot shows the multiple detection of viruses in co-infected tissue cultures. Two separate infections were done with the vaccine viruses as they appear in the two commercial multivalent preparations (serotypes 1, 3 and 4 in Lane I and serotypes 2, 6, 7 and 8 in Lane II).

### 3.3 Serotype-specific hybridization with field isolates

The universal primer set was derived from the sequence of the various reference strains that were isolated in the early 1960s (Koekemoer et al., 2000). Since RNA viruses are especially prone to mutations, we needed to establish whether the primers would be effective to detect current field isolates. A panel of 37 South African field isolates (Table 1) from different times of isolation was used to inoculate tissue cultures for dsRNA preparation. Amplification products were obtained from all the field viruses using the same reagents and procedures as used for the reference and vaccine viruses. The probes hybridized serotype-specifically to the reverse line blots and all the isolates were serotyped in accordance with the original VNT results. One result using field viruses that represent each of the nine serotypes is shown in Fig.3.

### 3.4 Serotyping directly from tissues

Spleen or lung necropsies are usually the way in which clinical samples are received in diagnostic laboratories for the confirmation and serotyping of cases where horses died and AHS is suspected. It was investigated whether the universal RT-PCR will be efficiently sensitive to prepare probes directly from the amount of virus dsRNA that is present in these tissue samples. Viral RNA was extracted from one lung and two spleen samples that were collected during a recent AHS outbreak. Genome segment 2-specific products could be obtained from all three RNA preparations by using the universal RT-PCR (Fig. 4a). Probes that were prepared from the same RNA hybridized specifically to the serotype 2 reverse line blot (Fig. 4b). The presence of non-specific amplification products (Fig. 4a) did not influence the sensitivity or specificity of the probes. The ability to serotype clinical organ samples directly without having to wait for virus isolation is a huge advantage for controlling the spread of outbreaks.

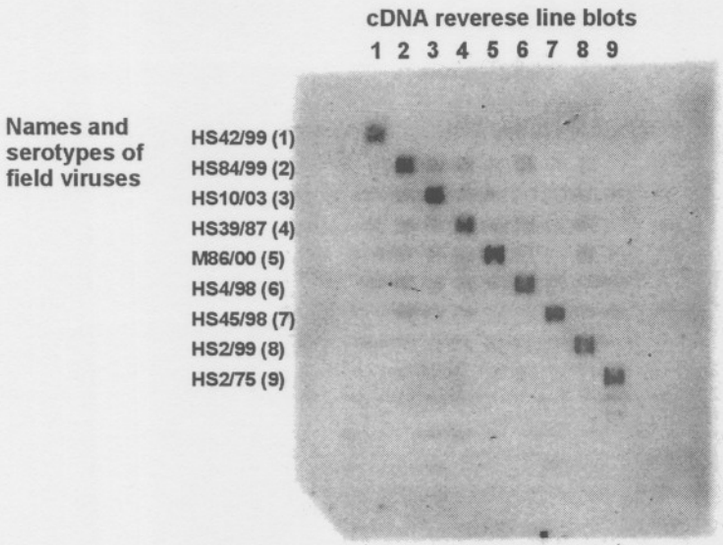


**Fig. 3.** RT-PCR and reverse line blot serotyping of a set of field isolate viruses (Table 1) that were selected to represent each of the nine serotypes of AHSV.

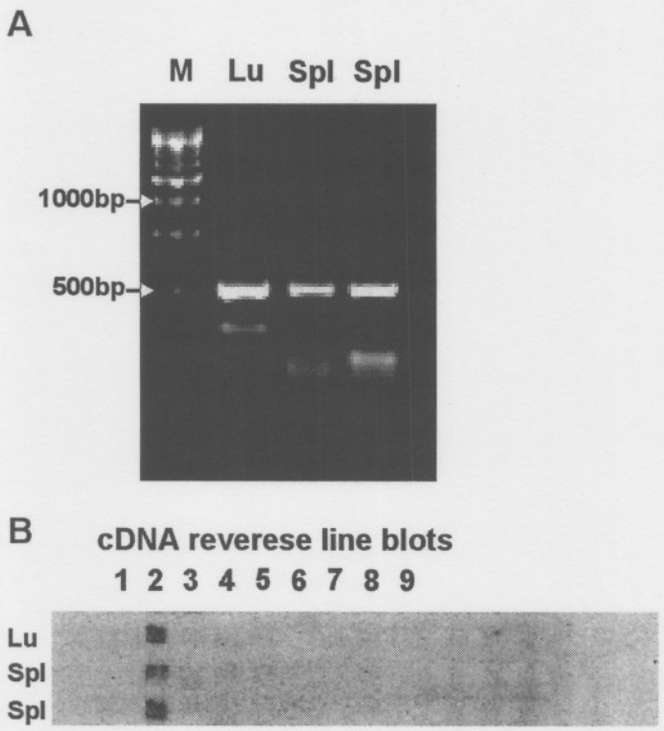


**Fig. 4.** (A) Universal RT-PCR amplification of genome segment 2-specific products from virus RNA that was extracted from one lung (Lu) and two spleen (Spl) necropsy samples from horses that died from AHS. (B) Reverse line blot hybridization with probes that were generated from the dsRNA extracted from the same lung and spleen samples.

## 4. Discussion

The spread of AHS outbreaks can be best controlled by rapidly vaccinating all horses in the affected region against the serotype that caused the outbreak. To efficiently implement such vaccination strategies, it is crucial to have quick access to reliable serotyping results. In this paper we further developed our previously described procedure for serotyping AHSV using genome segment 2 probes (Koekemoer et al., 2000). Its main drawbacks, namely the low sensitivity of the probes and the fact that virus RNA had to be blotted in nine copies and each hybridized with all the serotype-specific probes, were eliminated. This was achieved by designing a universal RT-PCR to amplify a part of genome segment 2 and use this amplification product in a single hybridization with a reverse line blot that contained the serotype-specific targets. The main advantage that was gained from this, is that serotyping can be done in one day directly from clinical samples from infected horses using a single RT-PCR and hybridization reaction. Additionally, it is the first and only method that can simultaneously identify all the serotypes in the case of mixed infections.

The use of RT-PCR to serotype AHSV that has been reported by Sialleau et al. (2000) has some similarity with the new method for serotyping developed in this study in the sense that both target part of the serotype-specific genome segment 2. The method of Sailleau et el. (2000) uses nine sets of primers, each amplifying a specific serotype. This method has the drawback that each dsRNA sample has to be submitted to nine separate RT-PCR and gel electrophoresis assays. The unique aspect of the RT-PCR and reverse line blot approach reported here, however, is that RT-PCR is used solely as a sensitive method of amplifying a part of genome segment 2 for use as a probe in a serotype-specific hybridization with the line blot. Because the RT-PCR amplification is not the serotype-specific step, primers specific for all nine serotypes can be mixed and used in a universal reaction to prepare probe(s) from the dsRNA of any serotype virus. This means that a single RT-PCR can be done to amplify genome segment 2-specific cDNA from any test sample. The method that we describe here does not to rely on the presence of an RT-PCR amplification product, but rather on nucleic acid hybridization to confirm the serotype. The problems that non-specific amplification products can introduce, are therefore eliminated.

To avoid the process of hybridizing each of the RT-PCR products with nine serotype-specific probes, the hybridization was carried out in the form of a reverse line blot hybridization. In this format the positions of the probe and the target are reversed. The major advantage is that one probe can be hybridized to multiple immobilized targets, such as the 9 serotype-specific genome segment 2 cDNAs, during a single hybridization reaction (Dattagupta et al., 1989; Saiki et al., 1989). Furthermore, by doing the hybridization in a checkerboard format (Sockransky et al., 1994), multiple probes can be hybridized simultaneously with all nine serotype-specific targets. The number of samples that can be typed in parallel is only limited by the physical format of the reverse line blots and makes the serotyping of multiple samples much easier and quicker to perform.

The chosen primer sequences seem to be well conserved since dsRNA from reference, vaccine, historical and field viruses, isolated over a 27 year period, were all amplified successfully. This confirms that despite the high sequence variation between genome segments 2 of the AHSV serotypes, variation between isolates of the same serotype seems to be much less. There were no instances where the specific RT-PCR amplification product was not obtained or where hybridization was not serotype-specific (Figs. 2, 3 and 4). Incorporation of digoxigenin labeled nucleotides did not adversely affect the efficiency of the RT-PCR or the serotype-specific hybridization of the probes. From these results it is concluded that the primer pool is indeed universal for all nine serotypes and that it is not necessary to use full-length genome segment 2 probes for serotype-specific hybridization. The choice of the 5' ~520 bp of genome segments 2 as a target for hybridization proved to be successful. The small amount of sequence variation that has been observed between isolates of one serotype of AHSV (Koekemoer et al., 2003) is not high enough to have an influence on the hybridization of the long (~520bp) probes.

We estimate the sensitivity of our RT-PCR to be in the same range as that of previous investigations for orbiviruses wherein specific products could be amplified from as little as 100 fg or $10^2$ genome segment copies (Dangler et al., 1990; Akita et al., 1993; Stone-Marschat et al., 1994; Aradaib et al., 1995) and from infected blood or tissues (Zientara et al., 1994; Stone-Marschat et al., 1994). The high sensitivity of the assay was demonstrated by performing serotypings directly from the small amounts of viral RNA present in infected tissue samples. This has the major implication that the process of virus

isolation can be avoided which markedly shortens the time needed to complete serotyping.

The purpose of designing the universal RT-PCR was that probe preparation can be performed using the same reaction for any serotype. It has, however, added the unique property to the assay that it can be used to generate multiple probes from a test sample that contains viruses of more than one serotype. This scenario is most likely after the vaccination of animals with multivalent live virus vaccines. With the experiments that were carried out, four different vaccine viruses could be serotyped simultaneously in one sample after a mixed inoculation. This is currently the only method by which the presence of more than one serotype of AHSV can be demonstrated with a single test in a single sample.

The two to three weeks needed to isolate and serotype AHSV with virological and serological methods have always hampered the rapid implementation of appropriate vaccination campaigns by adversely affecting both the extent and duration of outbreaks. In this report hybridization is again confirmed as a very reliable way to serotype any strain of AHSV. Coupled with the efficiency and sensitivity of RT-PCR, the reverse line blot hybridization can be used as a very quick and accurate diagnostic tool to serotype AHSV. Using this procedure, it is for the first time possible to have the serotype of an AHSV identified one day after a clinical specimen was received.

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# Chapter 4

## VP2-gene phylogenetic characterization of field isolates of African horsesickness virus serotype 7 circulating in South Africa during the time of the 1999 African horsesickness outbreak in the Western Cape

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Nucleotide sequence data was not published as part of this paper. The complete alignments of the serotype 7 and 3 gene-fragments are given in the Appendix.

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# VP2 gene phylogenetic characterization of field isolates of African horsesickness virus serotype 7 circulating in South Africa during the time of the 1999 African horsesickness outbreak in the Western Cape

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**Abstract**

We present the first VP2-gene phylogenetic analysis of African horsesickness (AHS) viruses within a serotype. Thirteen AHSV 7 isolates were obtained from cases that occurred in South Africa during 1998–1999, and three were historical AHSV 7 isolates. The goals were to start a database of isolates of known location and time of isolation and to determine if we could identify the origin of an AHS outbreak in the surveillance area in the Western Cape. We prepared full-length cDNA copies of the VP2-genes of the isolates. Nucleic acid sequence data of a 786 bp region was used to characterize the genetic relationships between the isolates. The nucleic acid identities between the isolates ranged from 95.5 to 100%. Isolates from common geographical regions grouped together. Characterization of field isolates revealed the presence of two AHSV 7 lineages in South Africa during this period. The grouping of the viruses into two clades accurately reflected the geographical groupings of the isolates. The average nucleic acid divergence between the clades was 4.3%. Within the clades the divergence was 0.5 and 0.1%, respectively. The data suggests that the AHS outbreak in the Western Cape could have been an incursion from the Kwazulu Natal Province.


*Keywords:* African horsesickness virus; VP2-gene; Phylogenetic analysis; Topotyping; Molecular epidemiology; AHSV 7

## 1. Introduction

African horsesickness (AHS) is a non-contagious, viral disease of equids with a high mortality rate in horses. Mules and donkeys are affected to a lesser extend and generally act as reservoirs. Blood sucking midges of the genus *Culicoides* are vectors for the aetiological agent, the *African horsesickness virus*, genus *Orbivirus*, family *Reoviridae*. The AHSV species consists of nine serotypes, AHSV 1–9, (McIntosh, 1958; Howell, 1962). To be fully protected against AHS, horses have to be immune against all nine serotypes. Strict regulating measures prohibit the national and international movement of horses, especially between endemic and AHS-free areas. In 1999 an outbreak caused by AHSV 7 occurred in an AHS-controlled area in South Africa, namely the surveillance area that surrounds an AHS-free zone of approximately 140 km$^2$ in the Western Cape Province. Cases were reported at several locations in and around Stellenbosch (Bell, 1999). The outbreak lasted from 21 March to 17 May 1999. At least 26 horses died from AHSV 7 infection as confirmed by serological testing. It was important to determine whether the outbreak was caused by the flare-up of a local strain or by the introduction of (a) viraemic animal(s) into the region. Since traditional virological methods cannot

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answer this question, we investigated whether genetic analysis of the VP2-genes of AHSV 7 isolates from 1998 to 1999 could be used to trace the origin of the outbreak.

The AHSV particle has a double layered capsid. The outer layer is composed of the two major proteins VP2 and VP5. The inner layer is made up of the major proteins VP3 and VP7 and constitutes the virus core that surrounds the minor proteins VP6, VP4 and VP1 as well as the double-stranded RNA genome of ten segments. The genome segments encode seven structural (VP1–VP7) and three non-structural (NS1–NS3) proteins. VP2 is the major protein on the outer capsid layer (Bremer, 1976; Van Dijk and Huismans, 1982; Lewis and Grubman, 1991) and carries the neutralizing epitopes (Burrage et al., 1993). Immune precipitation (Huismans and Erasmus, 1981) and cross-hybridization experiments with cloned bluetongue virus (BTV) and AHSV gene fragments established that VP2 is the major determinant of serotype for BTVs (Huismans and Cloete, 1987) and AHSVs (Bremer et al., 1990). These findings laid the bases for the current efforts to develop new molecular typing methods. For AHS, recent progress represents the development of a full set of cloned VP2-genes of each of the nine serotypes as type-specific probes (Koekemoer et al., 2000) and a VP2 specific RT-PCR procedure (Sailleau et al., 2000).

Molecular epidemiological and phylogenetic analyses of the orbiviruses are still in their infancy, largely due to technical difficulties in cloning and sequencing large dsRNA genes. However, the value of phylogenetic analysis in epidemiological studies of orbiviral diseases has been demonstrated through the analyses of different BTV isolates. Phylogenetic analysis of the conserved segment 3 (VP3-gene) of BTV, grouped isolates together reflecting their geographical origins (Gould, 1987; Pritchard et al., 1995). Phylogenetic analysis of BTV VP2-genes and proteins, grouped viruses from homologous serotypes together, irrespective of their geographical origin (Bonneau et al., 1999; Gould and Pritchard, 1990; Pritchard and Gould, 1995). However, when BTV isolates of the same serotype were analyzed, VP2 phylogenetic trees also clustered BTV isolates from common geographical origins together (Pritchard and Gould, 1995). Although the VP2 genes from different BTV isolates of the same serotype were found to be very closely related, they were sufficiently distinct to construct phylogenetic trees and to distinguish between different lineages of field isolate viruses (De Mattos et al., 1994a,b). It has also been suggested that divergent results that could be caused by reassortments of the smaller BTV genes, could be overcome by characterizing evolutionary relationships using the serotype-specific VP2-gene in phylogenetic analysis (De Mattos et al., 1994a). AHSV VP2 gene and protein comparisons have been made, and molecular relationships have been established between single isolates of AHSV serotypes 3, 4, 6 and 9 (Williams et al., 1998; Venter et al., 2000). However, phylogenetic analyses over a broad range of AHSV isolates within a serotype, based on nucleic acid sequence data from the VP2 genes, have not been reported.

This study was undertaken to determine whether it would be possible to generate genetic data for the molecular epidemiological characterization of AHSV 7 isolates from the 1999 Stellenbosch outbreak. We determined the degree of variance between segments 2 (VP2 genes) from different isolates of different geographical regions of South Africa spanning bases 837–1623 and investigated whether these variances could be used for topotyping. AHSV 7 isolates obtained from field cases in South Africa during 1998–1999, the AHSV 7 reference strain, two further historical AHSV 7 isolates as well as a midge isolate obtained during the Stellenbosch outbreak, were studied. The first aim was to determine if a correlation could be made between the inferred phylogeny of the VP2 genes and the known origin of the isolates. A second aim was to ascertain whether a genetic link could be drawn between viruses that were isolated during the Stellenbosch outbreak and other AHSV 7 viruses circulating in South Africa during the same time. This report describes the first phylogenetic analysis of AHSV VP2 gene sequence data over a range of field isolates of AHSV. We demonstrate that there were two distinct AHSV 7 lineages in South Africa during this period. The VP2 sequences of AHSV 7 isolates from Stellenbosch and Kwazulu Natal (KZN), locations which are approximately 1200 km apart, were very closely related and strongly suggests that the AHSV 7 outbreak in Stellenbosch could have been an incursion from KZN.

## 2. Materials and methods

### 2.1. Viruses and cells

All the field viruses were isolated at the Onderstepoort Veterinary Institute (OVI) from clinical samples of suspected AHS cases in South Africa. The names and origins of the virus isolates are given in Table 1. Viruses were isolated by inoculation of equine blood or homogenized organ samples into suckling mouse brains followed by three passages on BHK cell cultures. Serotyping was done through standard virus neutralization assays. The midge isolate was recovered from a collection of 500 parous *Culicoides imicola*, caught in a light trap set up in Stellenbosch on 27 March 1999. A homogenate of the insects was inoculated onto a BHK cell culture from which the virus was isolated. The 1981 and 1985 AHSV 7 isolates were part of the reference collection of historic AHSV isolates kept at the OVI Reference Center for AHS and were initially isolated

Table 1
Details of the origin and year of isolation of the field isolate viruses from which VP2-gene nucleic acid sequence data was used in the phylogenetic analysis

| Name | Year of isolation | Origin[a] |
|---|---|---|
| HS31/62 | 1962 | AHSV 7 reference strain |
| HS1/99 | 1999 | Horse, GA |
| HS18/99 | 1999 | Horse, GA |
| HS23/98 | 1998 | Horse, KZN |
| MP27/99 | 1999 | Midge Pool, WC |
| HS29/81 | 1981 | Horse, GA |
| HS29/85 | 1985 | Horse, GA |
| HS29/99 | 1999 | Horse, NW |
| HS40/99 | 1999 | Horse, NW |
| HS43/98 | 1998 | Horse, GA |
| HS45/98 | 1998 | Horse, GA |
| HS48/99 | 1999 | Horse, WC |
| HS58/99 | 1999 | Horse, WC |
| HS78/99 | 1999 | Horse, GA |
| HS82/99 | 1999 | Horse, GA |
| HS102/99 | 1999 | Horse, WC |

[a] GA, Gauteng Province; NW, North West Province; KZN, Kwazulu Natal; WC, Western Cape (Stellenbosch outbreak).

and passaged as described above and kept as a freeze-dried inventory. The AHSV 7 reference strain was first isolated in 1962. Vero cell cultures propagated in 75 $cm^2$ flasks were infected from virus stocks and harvested when clear CPE was visible through 80–100% of the culture. This seed stock was used to infect subsequent Vero tissue cultures for dsRNA preparation.

### 2.2. dsRNA preparation and RT-PCR

Viral dsRNA was isolated and purified from infected cells of three 75 $cm^2$ flasks with a commercial acid–phenol/guanidinium isothiocyanate based reagent (TRI-reagent, Molecular Research Center, Inc.) as described before (Koekemoer et al., 2000; Potgieter et al., 2002). The ssRNA and dsRNA were separated by means of differential LiCl-precipitation. Oligonucleotide primers, AHSV 7vp2vf (5′-gttgatggagggccaacaagagaag) and AHSV 7vp2vr (5′-cgtagacaccatcaatcggcactag), were designed from the sequence of the VP2-gene of the AHSV 7 reference strain (Potgieter et al., 2003) for the full length amplification of the approximately 3222 bp of the different VP2-genes. About 200 ng of total viral dsRNA was denatured in 5 μl by adding 1 μl 0.2 M methyl-mercury hydroxide (MMOH). The MMOH was reduced with 1.25 μl 1 M β-mercapto-ethanol before the RT-PCR. Reverse transcription was carried out at 50 °C for 40 min using 4 μl of the denatured RNA in a 20 μl reaction mixture containing 2 μl 2.5 mM dNTP-mix, 100 pmol of each of the forward and reverse primers, 4 μl cDNA-buffer (50 mM Tris–Cl, pH 8.3; 100 mM KCl; 5 mM $MgCl_2$) and 5 U of AMV reverse transcriptase (Promega). Four microliters of this cDNA-mixture was used for PCR amplification in a total volume of 50 μl. The PCR reaction mixture contained 5 μl 2.5 mM dNTP-mix, 100 pmol of each of the forward and reverse primers, 5 U of Ex-Taq polymerase (Takara) and 5 μl of the supplied 10 × reaction buffer. Thermal cycling conditions were as follows: initial denaturing of the cDNA was carried out for 3 min at 94 °C followed by 27 cycles of denaturing (30 s at 94 °C), annealing (30 s at 57 °C) and extension (2 min at 72 °C). After the final cycle an extension step of 10 min at 72 °C was added. The full-length VP2-amplicons were purified by electrophoresis on a 1% TBE agarose gel and extracted with a Qiaex gel extraction kit (Qiagen).

### 2.3. Cloning

The VP2-cDNA was digested with EcoRI and run out on an agarose gel. The 786 bp fragment was excised and purified with a Qiaex spin column (Qiagen) and resuspended in 10 μl of deionized water. The digested cDNA was added to dephosphorylated pBluescript SK+ vector (Stratagene) pre-cut with EcoRI and ligated using a Rapid DNA ligation kit (Roche).

### 2.4. Sequence and phylogenetic analysis

The cloned EcoRI-fragments were sequenced from plasmid DNA with an ABI PRISM 377 automated DNA sequencer using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v2.0 (Perkin–Elmer Applied Biosystems). The 786 bp sequences obtained from the cDNA clones of the field isolates were aligned to each other and to the VP2-sequences of the reference strains of AHSV 7 and AHSV 1 (Potgieter et al., 2003) and AHSV 3 (Vreede and Huismans, 1994) using the program CLUSTAL X (Thompson et al., 1997). Two clones from the midge pool isolate MP27/99 were sequenced from both ends. Identical nucleic acid sequences were obtained and this sequence was added to the analysis as a single entry. This multiple alignment was used to determine the pairwise $p$-distances from which the percentages of nucleic acid identity between the AHSV 7 isolates were calculated (Table 2). Two types of phylogenetic analyses, namely character state-based and distance-based methods, were implemented in determining the phylogenetic relationships between the sixteen AHSV 7 isolates. The programs were all part of the MEGA version 2.1-package of computer programs (Kumar et al., 2001). Distance matrixes were constructed using the Kimura-2-parameter model (Kimura, 1980). This model takes into account that transitions are statistically twice as likely to occur as transvertions when calculating the distance matrix. Algorithms based on the methods of Neighbor-Joining (Saitou and Nei, 1987) and the unweighted pair-group method using arithmetic mean,

Table 2
Percentage nucleic acid identities between the VP2-genes of AHSV 7 isolates in a 786 bp EcoRI fragment

| | HS102/99 | HS48/99 | HS58/99 | MP27/99 | HS23/98 | HS29/85 | Ref. strain | HS43/98 | HS45/98 | HS18/99 | HS82/99 | HS40/99 | HS29/99 | HS78/99 | HS1/99 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HS102/99 | - | | | | | | | | | | | | | | |
| HS48/99 | 100.0 | - | | | | | | | | | | | | | |
| HS58/99 | 100.0 | 100 | - | S | | | | | | | | | | | |
| MP27/99 | 99.9 | 99.9 | 99.9 | - | | | | | | | | | | | |
| HS23/98 | 99.9 | 99.9 | 99.9 | 99.7 | - | | | | | | | | | | |
| HS29/85 | 98.9 | 98.9 | 98.9 | 98.7 | 98.7 | - | | | | | | | | | |
| Ref. strain | 97.3 | 97.3 | 97.3 | 97.2 | 97.2 | 98.0 | - | | | | | | | | |
| HS43/98 | 95.8 | 95.8 | 95.8 | 95.7 | 95.7 | 96.2 | 97.2 | - | | | | | | | |
| HS45/98 | 95.7 | 95.7 | 95.7 | 95.5 | 95.5 | 96.1 | 97.1 | 99.6 | - | | | | | | |
| HS18/99 | 95.9 | 95.9 | 95.9 | 95.8 | 95.8 | 96.3 | 97.1 | 99.6 | 99.5 | - | | | | | |
| HS82/99 | 95.8 | 95.8 | 95.8 | 95.7 | 95.7 | 96.3 | 96.9 | 99.5 | 99.4 | 99.9 | - | N | | | |
| HS40/99 | 95.7 | 95.7 | 95.7 | 95.5 | 95.5 | 96.1 | 96.8 | 99.4 | 99.2 | 99.7 | 99.6 | - | | | |
| HS29/99 | 95.8 | 95.8 | 95.8 | 95.7 | 95.7 | 96.2 | 96.9 | 99.5 | 99.4 | 99.9 | 99.7 | 99.8 | - | | |
| HS78/99 | 95.7 | 95.7 | 95.7 | 95.5 | 95.5 | 96.1 | 96.8 | 99.4 | 99.2 | 99.7 | 99.6 | 99.5 | 99.9 | - | |
| HS1/99 | 95.7 | 95.7 | 95.7 | 95.5 | 95.5 | 96.1 | 96.8 | 99.4 | 99.2 | 99.7 | 99.6 | 99.5 | 99.9 | 99.7 | - |
| HS29/81 | 96.4 | 96.4 | 96.4 | 96.3 | 96.3 | 96.8 | 97.8 | 98.3 | 98.2 | 98.2 | 99.1 | 98.0 | 98.1 | 98.0 | 98.0 |

The nucleic acid identities between the isolates within the N and S clades (Figs. 3 and 4) are boxed. Bold values indicate where the lowest nucleic acid identities between two isolates were observed.

UPGMA (Michener and Sokal, 1957) were used to construct phylogenetic trees from the distance matrix data. The character state-based method of maximum parsimony as applied to genetic data (Fitch, 1971) was used to produce a tree from the multiple alignments of the sequences. To assess the robustness of the groupings, the trees were tested by performing bootstrap resampling on 1000 replicates of the data.

## 3. Results

The aim of this investigation was to start an AHSV 7 VP2 nucleic acid sequence data set and determine whether it could serve as a basis for molecular epidemiology to determine the origin of an AHSV 7 outbreak in the AHS surveillance area during 1999. During 1998–1999, 28 isolations of AHSV 7 were made at the OVI. For the purpose of this study representative samples were selected from all parts of South Africa (Fig. 5) namely: two North West Province isolates, a single KZN isolate and six isolates from nine that originated in the Gauteng Province. Apart from the outbreak in the Western Cape, these were the only South African AHSV 7 isolates obtained during 1998–2000. From the Stellenbosch outbreak, one midge pool and 15 horse isolate viruses were available. From these, three horse isolates and the midge isolate were used.

### 3.1. RT-PCR, cloning and RFLP analysis

The first goal was to prepare full-length cDNAs from the selected isolates and do a pilot comparative restriction enzyme analysis. RT-PCR for all the virus isolates, with primers designed from the 5′ and 3′ terminal sequences of the AHSV 7 reference strain gave VP2-cDNA amplicons of an equivalent size to the 3222 bp of the reference strain gene. The full-length VP2-cDNA products of six of the isolates and the pilot comparative restriction enzyme analysis of these six isolates are shown on the agarose gels in Fig. 1. The isolates were selected as follows: two from the 1999 Stellenbosch outbreak (HS58/99 and HS102/99), three made during 1998–1999 in South Africa from the Gauteng (GA) and North West (NW) provinces (HS29/99, HS 78/99 and HS 45/98) as well as the reference strain virus used in this study. The EcoRI restriction profile of the cDNA amplicons of the VP2 genes of all the isolates were

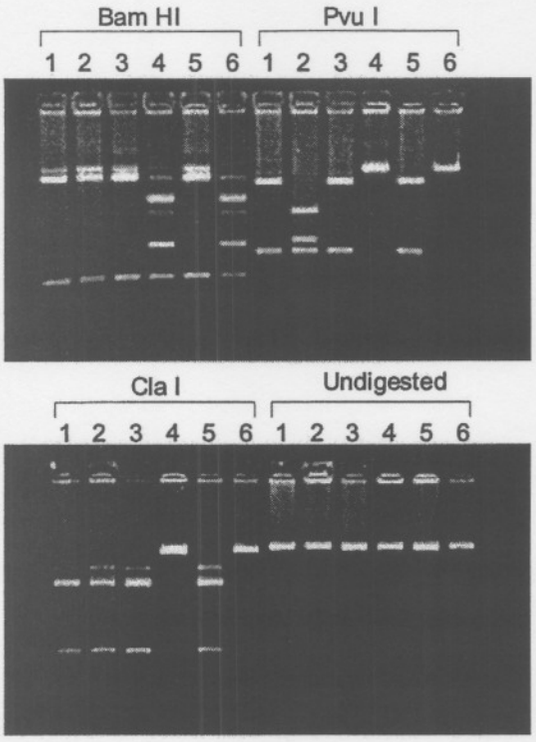


Fig. 1. Agarose gel of restriction fragment length patterns of full-length cDNA of VP2-genes from six different AHSV 7 isolates digested, respectively, with BamHI, PvuI, ClaI and undigested cDNA. Lanes 1, 3 and 5 contained cDNA from isolates of the northern provinces: Lane (1) HS29/99; (3) HS45/98; (5) HS78/99. Lanes 4 and 6 contained cDNA from the Stellenbosch isolates: Lane (4) HS58/99; (6) HS102/99. Lane (2) cDNA of the reference strain. Undigested cDNA was loaded in the same order.

identical (data not shown). Three restriction enzymes (BamHI, ClaI, and PvuI) demonstrated heterologies in the sequences of the VP2-genes (Fig. 1). A pattern emerged that divided the viruses into three groups, each with a distinct set of fragment lengths. The first group contained the two isolates from the 1999 Stellenbosch outbreak, the second contained only the reference strain and the third consisted of the three isolates made from the GA and NW provinces. This established that there was sequence variation between the VP2 genes of this set of AHSV 7 isolates. The variations as observed in the restriction fragment patterns, corresponded to the differences in the geographical and chronological origins of the isolates. These initial findings prompted us to continue the investigation by cloning, sequencing and comparing a part of the VP2 genes from the full set of 16 AHSV 7 isolates listed in Table 1.

### 3.2. Sequence and phylogenetic analyses

As all the isolates under investigation were of the same serotype, we decided not to concentrate exclusively on the part of the gene proposed to encode the neutralizing epitope(s) of the virus. An RFLP-assay was done to determine if sequence variations could indicate a particular part of the gene to be more variable between the AHSV 7 isolates. Since results showed that sequence variations occurred throughout the length of the gene (Figs. 1 and 2), we used conserved restriction enzyme sites to clone a uniform part of the gene for sequencing and phylogenetic analysis. Two conserved EcoRI-sites, at positions 837 and 1627 (Fig. 2), made it possible to subclone and sequence a 786 bp region from all 16 AHSV 7 isolates. This fragment spans most of the region encoding amino acids 200–400, which most likely contains the neutralization-specific epitopes (Bentley et al., 2000; Martínez-Torrecuadrada and Casal, 1995; Martínez-Torrecuadrada et al., 2001; Venter et al., 2000). The GenBank accession numbers of the nucleic acid sequences from the field isolates are AY 159940–AY 159954. Out of the 786 bases, 55 were found to be variable. From the pairwise alignments the percentage nucleic acid identities between the isolates were calculated (Table 2). The nucleic acid identities between the AHSV 7 isolates ranged from 95.5 to 100%. The highest divergence was observed between the midge pool (MP27/99) and KZN (HS23/98) isolates, respectively, and a group of isolates (HS45/98, HS78/99, HS1/99, HS40/99) made during 1998–1999 from the GA and NW provinces (Table 2). There was 100% nucleic acid sequence identity between the VP2 genes of the viruses obtained from three separate clinical samples submitted during the Stellenbosch outbreak. This sequence differed from that of the reference strain in 21 positions. The midge pool isolate differed from the three Stellenbosch horse isolates by a single A–G transition that leads to a Thr-Ala amino acid substitution. The sequence of the KZN isolate (HS23/98) also differed from the Stellenbosch horse isolates by a single base, in

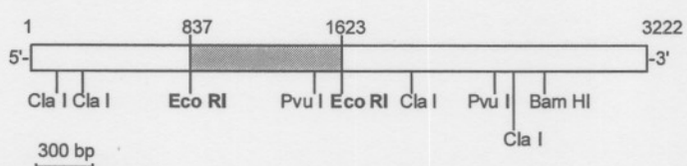


Fig. 2. Restriction map of the VP2-gene of the AHSV 7 reference strain indicating the recognition sites of the restriction enzymes used in the RFLP-assay. The 786 bp EcoRI-fragment (shaded area from 837 to 1623 bp) from each of the isolates was cloned and sequenced for use in the phylogenetic analysis.

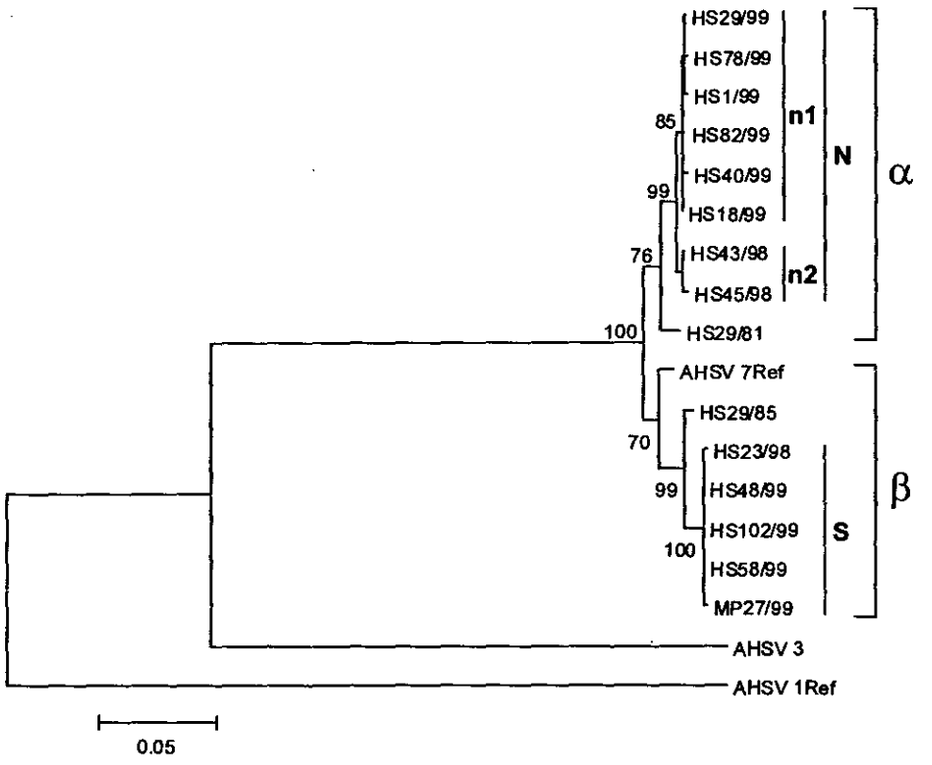


Fig. 3. Phylogenetic tree constructed from nucleic acid sequence data of the 786 bp EcoRI-fragment of the VP2-gene of AHSV 7 isolates. Genetic distance data was used with the neighbor-joining algorithm to construct the tree that was rooted with VP2-gene sequences form AHSV 1 and AHSV 3. Bootstrap confidence levels of higher than 70% are shown on the nodes. The scale bar indicates the genetic distance between the sequences from the different taxa.

this case a synonymous G–A transition. Of the 55 sites where substitutions were observed, only four were transversions, all A–T. The VP2-gene nucleic acid identities between the average of the AHSV 7 group and AHSV 3 and AHSV 1 were 68.4 and 58.7%, respectively.

Two phylogenetic trees were constructed from a distance matrix calculated using the Kimura-2-paramter model. The distance data sets were used with the neighbor-joining and UPGMA algorithms to construct the phylogenetic trees. The resultant phylogenetic trees were essentially the same: two monophyletic groups (clades) α and β were formed, containing all the AHSV 7 isolates. Fig. 3 shows the neighbor-joining tree. The α clade was made up of isolates from the GA and NW provinces and a historical isolate, HS29/81. The β clade contained two historic isolates namely, the AHSV 7 reference strain and HS29/85 together with the midge pool and the three horse isolates from Stellenbosch. Also included in this clade was the isolate from KZN, HS23/98. When only the recent (1998–1999) AHSV 7 isolates were considered, two smaller clades could be identified. The first contained isolates from the two northern provinces of Gauteng and North West, and was labeled N and the second, labeled S, contained the Stellenbosch horse and midge pool isolates and the KZN isolate. These two groupings occurred in the phylogenetic tree with a very high bootstrap confidence level of 99 (Fig. 3). A further division into clusters n1 and n2 could be seen within the N clade. In this case isolates made during the same years grouped together in both the trees. VP2-gene nucleic acid sequence data from AHSV 1 and AHSV 3 were used to form an outgroup.

A maximum parsimony analysis was also conducted, and the resultant cladogram shows the 50% majority rule consensus tree (Fig. 4). The topology was almost identical to that produced by the distance-based algorithms. All the AHSV 7 isolates were again divided into two clusters α′ and β′, with the 1998–1999 isolates in the same two distinct clades, N and S. The n1 and n2 division between the 1998 and 1999 isolates also re-appeared with a bootstrap consensus value of 92. The only inconsistency with the distance based methods was the placement of the historic isolate, HS29/81, on the same branch as the reference strain. This resulted in an exchange of one terminal taxon between the α and β groups as they appeared in the distance-based trees.

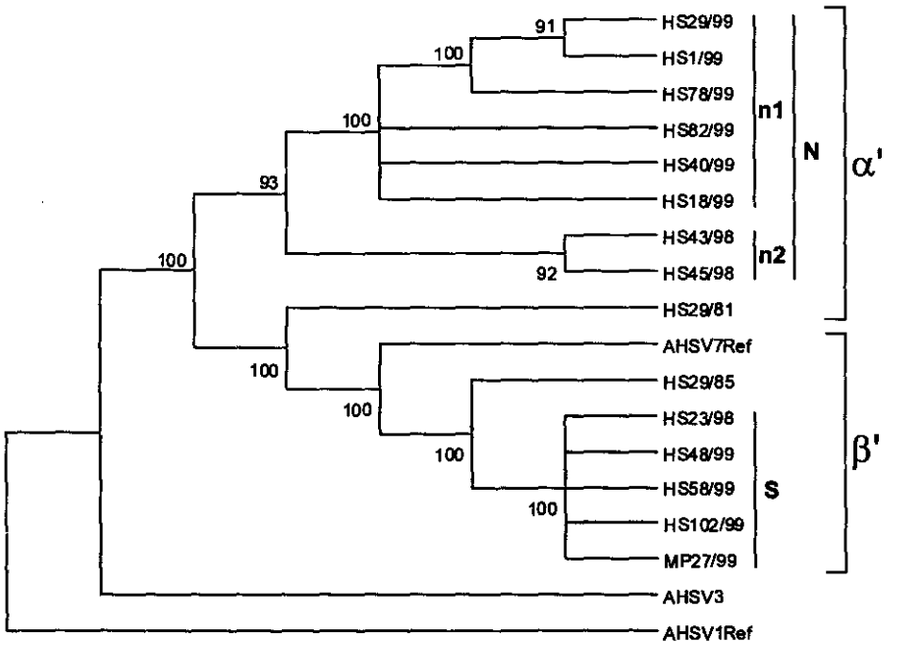


Fig. 4. A cladogram of AHSV 7 isolates showing the majority rule consensus tree produced from all the most-parsimonious trees as calculated by the method of maximum parsimony from the nucleic acid sequences of the 786 bp EcoRI-fragment of the VP2-genes of the isolates. The tree was rooted by the same outgroup as the distance-based tree in Fig. 3. Bootstrap consensus values were placed on all the nodes.

## 4. Discussion

In this study, we have generated nucleic acid sequence data from a 786 bp region of the VP2-genes of 13 recent AHSV 7 field isolate viruses and three historical AHSV 7 isolates of known origin. The sequences were used to establish the genetic relationships between the different viruses by means of a phylogenetic analysis.

In the literature different strategies have been reported to determine the phylogenetic relationships between viruses in the orbivirus genus. Apart from initial studies demonstrating that BTV segment 3 (VP3 gene) nucleic acid sequences makes it possible to topotype isolates, other BTV genome segments have been investigated. These include the VP7 gene (Wilson et al., 2000; Bonneau et al., 2000), the VP6 gene (De Mattos et al., 1996) and the NS3 gene (Bonneau et al., 1999; Pierce et al., 1998). Results mostly showed no correlation of the phylogenetic groupings with the origin of the viruses.

Phylogenetic analyses of AHSVs have been more limited and mostly focused on the small NS3-gene (segment 10). In the AHSV species, the NS3 gene is the second most variable gene after the VP2 gene (Bremer et al., 1990; Van Niekerk et al., 2001). Segment 10 (NS3 gene) sequences and evolutionary relationships have been compared across a range of AHSV isolates and serotypes (De Sá et al., 1994; Sailleau et al., 1997; Van Niekerk et al., 2001). Several isolates of AHSV 6 and AHSV 4 were compared, and a variation was found to exist within each of these serotypes. A closer relationship was observed between AHSV 4 isolates from Spain and Marocco than between virulent and vaccine strains of serotype 4. This was taken as an indication that the virus initially present in Spain subsequently caused AHS in Marocco (Sailleau et al., 1997). More recently, the variation between the NS3 amino acid sequences of several South African AHSV isolates was determined (Van Niekerk et al., 2001). The same relationships were observed as described by De Sá et al. (1994) and Sailleau et al. (1997), with the exception of one AHSV 8 isolate that grouped differently than expected. The reason for this is given as possible gene rearrangements that had taken place during mixed wild infections (Van Niekerk et al., 2001).

When we analyzed the VP2 gene sequences of the 13 AHSV 7 field isolates from 1998 to 1999 in this study, nucleic acid identities were found to range between 95.5 and 100%. This is comparable to the range of 93.8–100% observed within two BTV serotypes (De Mattos et al., 1994a,b). Compared with the VP2-sequence from AHSV 1 and AHSV 3, the average nucleic acid identities of the AHSV 7 group were only 59.7 and 68.4%, respectively. This shows that, as for BTV, the VP2-gene sequence diversion within a serotype is much lower than that between serotypes, implying that AHSV VP2-gene data will also only be effective in studying molecular epidemiology of field isolates within the

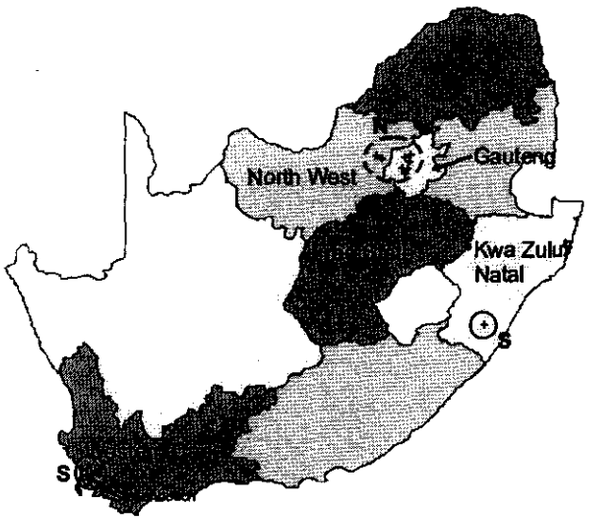


Fig. 5. Provincial map of South Africa showing the locations of the cases of AHSV 7 infection from which nucleic acid sequence data was used in this study. The Gauteng and North West province cases made up clade N and the KZN and Stellenbosch cases made up clade S. The *, symbol shows where the midge pool was trapped.

same serotype. The subsequent phylogenetic analysis showed that although small, the divergence within the VP2-genes proved to be useful in determining the evolutionary relationships between the different isolates. The three methods that we used for constructing phylogenetic trees, all gave trees with near identical topologies. The bootstrap confidence levels on the major nodes were very high. Furthermore, the grouping of the viruses into the N and S clades (Figs. 3 and 4) accurately reflected the geographical groupings of the isolates (Fig. 5). Together, this authenticates the phylogenetic trees as a true representation of the evolutionary relationships between the viruses. Small disparities did occur between the trees in the placement of some of the terminal taxa from geographically close areas where the nucleic identities between the isolates were expectantly very high. These changes do not influence any of the monophyletic groupings (N, n1, n2 and S) that are considered to be single topotypes. The only major contradiction between the topologies is the placement of HS29/81. This is due to the fact that maximum parsimony analysis only utilizes data from parsimony-informative sites on aligned nucleic acid sequences. Far less information is, therefore, available to construct the phylogenetic tree and especially when the different taxa are as closely related as in this case, inaccuracies may occur.

The division of the sequences into two distinct clusters, α and β, denotes that two lineages of AHSV 7 were present in South Africa during the time when the field samples were obtained. There was a large genetic separation between the N and S clades with an average nucleic acid divergence between the two clades of 4.3%. Within the N and S clades it was only 0.5 and 0.1%, respectively. This provides a clear-cut division between the two clades, making it easy to place any isolate with certainty into one of the two clades that represent two different topotypes of AHSV 7. It is also noteworthy that even though the elapsed time between the isolation of the viruses in clusters n1 and n2 was small, enough genetic divergence had occurred for these two groups to be distinguishable in all the phylogenetic trees. This result further strengthens the conclusion that an accurate description the true phylogeny of the isolates had been achieved.

The virus isolates from horses that died over the 31 days of the Stellenbosch outbreak did not show any genetic variation. This suggests that the outbreak started as the result of a single case of AHSV 7 infection. As expected, the isolate from the midge pool grouped together closely with the viruses from the horses, appearing in the same topotype. A very close relationship was detected between the VP2 genes of the Stellenbosch isolates and that of the 1998 isolate from KZN. Although the KZN case was geographically well isolated from the outbreak in the Western Cape, its nucleic acid sequence was almost identical to that from the Stellenbosch isolates. Previously the South African Veterinary services suspected that AHSV 7 was introduced from the Free State province (Bell, 1999), but this new evidence and the fact that AHSV 7 did not occur in the Free State province from 1998 to 2000, strongly suggests that the virus was introduced from KZN between March 1998, when this case occurred and February 1999 when the Stellenbosch outbreak started.

We conclude that the VP2 gene phylogenetic approach can be useful in molecular epidemiology to establish the genetic relationship between AHSV isolates, assign isolates within a serotype to particular topotypes and assist in tracing an outbreak to a source. These findings also emphasize the need for setting up a comprehensive molecular database from historical as well as recent field isolates by which outbreaks can be linked to previous or concurrent cases based on genetic evidence. Such information will be useful to monitor the presence and spread of AHSV and point out weaknesses in movement control systems.

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# Chapter 5

## Concluding discussion

African horsesickness is one of the few veterinary diseases that are classified by the OIE in the group of transmissible diseases that (i) have the potential for very serious and rapid spread irrespective of national borders, (ii) are of serious socio-economic or public health consequence and (iii) are of major importance in the international trade of animals and animal products. It is endemic in southern Africa where outbreaks occur on an annual basis. As part of the global epidemiological surveillance of the disease, all cases have to be referred to one of the OIE international reference centers for identification and serotyping of the virus. Currently, serotyping is done by performing laborious virus neutralization assays on tissue culture and the process takes two to three weeks to complete. The need for developing new protocols that speed up serotyping has long been identified and efforts to generate nucleic acid based serotyping methods for AHSV have been ongoing ever since the advent of recombinant DNA technology. The fact that genome segment 2 shows the highest degree of variation between serotypes (Bremer et al., 1990) and that it encodes the serotype-specific neutralizing epitopes (Burrage et al., 1993), makes it the obvious choice for the development of a nucleic acid based serotyping method. Although modern molecular techniques have been used to establish nucleic acid based diagnostic methods for many infectious diseases, difficulties in cloning the large genome segment 2 dsRNAs have thus far hampered similar progress for the orbiviruses. A recent breakthrough in our laboratory has been the development of a sequence-independent method for the cloning of large dsRNA molecules (Potgieter et al., 2002) that was used to obtain genome segment 2 clones from all nine AHSV serotypes (Potgieter et al., 2003). The availability of these clones was the starting point for this investigation to determine the feasibility of developing them into serotyping reagents. During the course of this study two new genome segment 2 based serotyping methods were developed, both of which are considerably faster than the existing serological procedure: serotype-specific probing requires 4 days and RT-PCR reverse line blot serotyping can be performed within 1 day.

The first goal of this study was to determine if the nine AHSV genome segment 2 clones could be utilized to distinguish between the serotypes of AHSV. Experiments were carried out during which probes generated from these clones were hybridized to virus RNA of the nine serotypes (Chapter 2). Initial results showed that, under high stringency conditions, the nine full-length genome segment 2 probes hybridized exclusively to RNA targets of their corresponding serotypes. This established proof of concept for the feasibility of developing serotype-specific probes for all the serotypes. It indeed provided, for the first time, a method for genomic serotyping of a complete orbivirus species with nucleic acid probes. The hybridization method made it possible to serotype virus isolates considerably faster than with serological typing. Apart from being a quicker method, it also has the advantage that it does not require any reagents to be generated in experimental animals as is the case with serotype-specific antisera.

These findings were followed up by investigations to improve the sensitivity of the assay. The sensitivity of probe hybridization is limited by the amount of immobilized target material that is available for binding. In this case the targets are virus dsRNA samples that are prepared from infected tissue cultures and blotted on the membrane. Genome segment 2 probes could detect 18.7 ng or 37.5 ng total genomic RNA respectively using radio-labeling and digoxigenin labeling methods. This meant that even at high multiplicities of infection the virus has to be amplified for at least 16 hrs on tissue culture to be detectable with the probes (Chapter 2). The aim was to overcome this limitation by RT-PCR amplification of segment 2-specific cDNA from the virus RNA and use this for hybridization in place of genomic RNA. Nine sets of primers were designed for the specific amplification of the first ~520 bp of genome segment 2 of all the serotypes of AHSV. To avoid using nine individual RT-PCRs to cover all serotypes, it was decided to test a combination of all the primer sets in a single RT-PCR. In this way a single reaction can be used to amplify a specific part of genome segment 2 from any serotype. Because of the high degree of genome segment 2 sequence variation between the different serotypes, the number of primers could be limited to only seven sense and nine antisense primers (Table 2, Chapter 3). To make sure that the primers could be used together they were designed to have annealing temperatures that are within a narrow range. This resulted in an RT-PCR that is universal for all 9 serotypes that could be used to amplify a corresponding part of

genome segment 2 from any AHSV under the same reaction conditions with one set of reagents (Chapter 3).

However, instead of carrying out the serotyping by blotting the RT-PCR amplicons and hybridizing each with nine probes, it was decided to make use of the advantages of reverse line blot hybridization. In this format the probe is prepared from the sample and hybridized to targets that are immobilized in specific locations on a membrane (Dattagupta et al., 1989; Saiki et al., 1989). Reverse line blot hybridization has been successfully used to design genotyping assays for medically important viruses (Gravitt et al., 1998; Vinje and Koopmans, 2000) and for the simultaneous detection of different strains of haemoparasites (Gubbels et al., 1999) and *Mycobacterium bovis* (Aranaz et al., 1996) in cattle.

Several virus RNA samples were used as templates for the RT-PCR to generate labeled probes that were hybridized to genome segment 2 cDNA of the nine serotypes of AHSV. Probes could be prepared in this way from virus RNA of all nine serotypes using the universal primer set. As with the full-length genome segment 2 probes, the hybridization of the RT-PCR generated probes was consistently serotype-specific (Chapter 3). It was further shown that serotype-specific probes, spanning the same part of genome segment 2, could be amplified from a range of field isolates of all nine serotypes as well as all the vaccine strain viruses, using the same universal primer set and reaction conditions. This accomplishment represented the first application of reverse line blot hybridization for the serotyping of a complete orbivirus species.

RT-PCR can improve the sensitivity of any nucleic acid detection by the specific amplification of very small amounts of target material. RT-PCR based methods for orbiviruses have been described that can detect as little as $10^2$ genome copies or 100 fg virus RNA (Dangler et al., 1990; Akita et al., 1992; Aradaib et al., 1995; Stone-Marschat et al., 1994). In this study, RT-PCR was used to amplify genome segment 2 cDNA from virus RNA and label for use as a probe. The sensitivity of the method was increased to the extent that it can be used to serotype directly from RNA isolated from infected tissues. Clinical specimens from suspected AHS cases that are send for virus identification and serotyping are usually in the form of spleen, lung or lymph node necropsy samples. This new method was successfully used to serotype 2 cases directly

from virus RNA that was isolated from spleen and lung tissue samples. This has the advantage that all virus isolation and amplification steps can be avoided, leaving only RNA extraction, probe preparation and hybridization. All these procedures can be carried out in one day, making this the fastest method that is currently available to serotype AHSV.

Reverse blotting lends an added benefit to this procedure. It is the only single assay developed to date that can be used to simultaneously detect the presence of multiple AHSV serotypes in one sample. VNTs are limited in this respect by the cross-neutralization that occurs between different serotypes (McIntosh, 1958). A serotype-specific anti-serum will give the strongest neutralization of a homologous serotype virus, but it will also neutralize other serotypes to varying extends. It can consequently not be used to exclude the presence of any other serotype viruses.

In the literature an RT-PCR technique for the serotype-specific detection of AHSV has been described. It makes use of nine RT-PCR primer sets that will each only amplify a part of genome segment 2 of a specific serotype of (Sailleau et al., 2000). These RT-PCRs could be used to detect more than one serotype in a sample, but it has the drawback that would require the sample to be tested with nine individual RT-PCRs respectively. With the new method that is described in this study, the RT-PCR probe generation step is not serotype-specific and it will concurrently amplify probes from virus RNA of all serotypes that are present in the sample. During the ensuing hybridization step, the presence of multiple serotypes in the sample will be indicated (Chapter 3).

Although multiple serotype infections do not normally occur in field cases, there are potential diagnostic applications. The current AHS vaccine is a multivalent live virus preparation containing seven serotypes of AHSV that are administered in two batches. These viruses can all multiply and be present in clinical samples when an animal dies shortly after it has been vaccinated. Using the new technique developed during this study, multiple vaccine strain viruses can be indicated in a sample with a single assay (Chapter 3). It also has specific application in assessing the quality of virus stocks. Single serotype reference virus preparations are used for making serological reagents. If these are contaminated with a virus from another serotype, the accuracy of the test is

jeopardized. This method has been utilized to indicate the presence of small amounts of contaminating AHSV 3, that could not be detected by VNTs, in stocks of AHSV 7 preparations (data not shown).

A drawback of the reverse line blot method is that it requires post RT-PCR hybridization and probe detection to be carried out separately. The next step that is envisaged in the streamlining of this assay, is to replace these individual procedures with an amplification and probe hybridization reaction in one tube. With real-time PCR technology (Heid et al., 1996) serotype-specific hybridization probes could be added to the reaction mixture and the binding of the probes to the genome segment 2 amplification product can be monitored as the PCR proceeds. The current set of universal primers can be used for the amplification of the target fragments, and it would only require the design of serotype-specific hybridization probes.

The new nucleic acid-based serotyping methods developed during this study have been used very successfully on an experimental basis in parallel with serological typing. The next step is to fully evaluate and validate this assay for routine diagnostic use. It is important to keep in mind that even though serotype-specific hybridization with conventional genome segment 2 probes (Chapter 2) is less sensitive and takes longer than reverse blot serotyping with RT-PCR generated probes (Chapter 3), it has the advantage that it does not depend on the conservation of the primer sequences and might therefore be more useful for serotyping samples from widely separated geographical regions.

The second major goal of this study was to initiate the first AHSV genome segment 2-based molecular epidemiological data set and investigate whether AHSV genome segment 2 phylogenetic analysis can be of practical use. A comprehensive body of traditional epidemiological data on AHS has been acquired over the past century in South Africa and safe and effective vaccines are available. However, being situated in an endemic region, South Africa is still plagued by recurrent outbreaks of this disease. Regions particularly prone to outbreaks are those where outbreaks have not occurred for a few seasons and people tend to vaccinate inconsistently. Furthermore, vaccination is prohibited in certain areas of the Western Cape Province that have been designated as

AHS-surveillance and free zones. When an infected animal(s) are inadvertently brought into one of these areas and the conditions are such that the vector is abundant, it usually leads to extensive outbreaks. In some cases these outbreaks can involve hundreds of animals (Eastern Cape, 2000 and Kwa-Zulu Natal, 2003; Unpublished reports) or have a severe negative impact on international trade (Stellenbosch, 1999, outbreak in the AHS-surveillance zone, Reported by Bell, 1999). The spread of disease outbreaks can never be accurately predicted or prevented. Underlying to these problems are the uncertainty about the overwintering mechanisms of the virus and a lack in the ability to trace the routes by which the disease is spread.

A genome segment 2 sequence data set (Appendix) was generated from field isolated viruses and used in a phylogenetic analysis to determine its usefulness in molecular epidemiology. A group of AHSV 7 field and historic isolates from South Africa were used in this analysis. Notably, amongst these were isolates from an AHSV 7 outbreak that occurred during 1999 in the AHS-controlled area in Stellenbosch, South Africa (Bell, 1999 ). It was very important to determine the origin of the strain that caused this outbreak. Since traditional virological methods could not answer this question, we investigated whether genetic analysis of genome segment 2 of AHSV 7 isolates from 1998 to 1999 could be used for this purpose. The phylogenetic analysis showed that the divergence within genome segment 2 proved to be useful in determining the evolutionary relationships between the different isolates. The virus isolates from horses that died over the 31 days of the Stellenbosch outbreak did not show any genetic variation. This suggests that the outbreak started as the result of a single AHSV 7 incursion. The isolate from the midge pool grouped together closely with the viruses from the horses, appearing in the same topotype. A very close relationship was detected between the Stellenbosch isolates and a 1998 isolate from KZN. Although the KZN case was separated from the outbreak in the Western Cape, its genome segment 2 sequence was almost identical to that of the Stellenbosch isolates (Chapter 4 and Appendix). The suspicion that the virus that caused the Stellenbosch outbreak originated in the Free State province (Bell, 1999) was disproved. The results from this study indicated that the virus that was present in KZN during 1998, was subsequently introduced to the Western Cape and caused the outbreak in the AHS-controlled area.

The conclusion that was reached was that the mutations that are accumulated in genome segment 2 of AHSVs over time, can be used to reflect the evolutionary history of the virus. The replication bottlenecks that the virus undergoes during overwintering, means that virus populations can be distinguished after even one season. Distinct lineages of viruses can be identified within a single serotype of AHSV. These lineages, as they are identified by phylogenetic groupings, are a reflection of the geographic and historic groupings of the viruses. It, therefore, provides for the topotyping of AHSV isolates by means of molecular analyses of genome segment 2. The most important application of this lies in its use to trace the origin of a virus back to genetically related strains that have been isolated from other localities.

With more investigations like these, the routes by which AHS is spread can be pointed out and preventative measures can be put into place. The impact that molecular epidemiological investigations can have, however, depends heavily on the amount of data that exists from different isolates. The more complete the sequence database, the more accurate and useful the topotyping and subsequent epidemiological findings will be. This study can be used as a motivation for the establishment and continuous upkeep of a database with nucleic acid sequence data to be used together with other epidemiological data.

In conclusion, results from this study have (i) demonstrated the feasibility of developing AHSV genome segment 2-based serotyping methods, (ii) led to the development of the first nucleic acid based serotyping procedures for all nine serotypes, (iii) generated the first genome segment 2 phylogenetic data for AHSV and (iv) generated the first example of the advantage of a molecular epidemiological approach to provide definitive data to shed light on the origin of an AHSV outbreak. Molecular analysis and applications of the complete set of cloned genome segments 2 of all nine AHSV serotypes have been used to achieve both aims set at the beginning of this study. It has been determined that genome segment 2-based nucleic acid hybridization can be used to rapidly and accurately serotype AHSV isolates, providing an effective and more rapid alternative to serological typing. With further refinement and validation of the method, it could in future be easily used in routine diagnostics applications. The knowledge gained in the phylogenetic characterization of the genome segment 2 nucleic acid sequence data, showed that it has a very important application in the study

of the epidemiology of AHSV in the animal host and vector species. The biggest benefit lies in the gaining of a reliable way of topotyping virus isolates. After expansion of the molecular database, this information can be used to increase the effectiveness of current regulatory measures and hopefully add in a positive manner to disease control efforts. In future, the use of these new serotyping procedures in conjunction with comprehensive molecular epidemiological data should significantly speed up AHSV diagnostics and contribute to more effective prevention, control and surveillance systems for AHS. In time, the new technologies will hopefully also make it possible to revise the zoosanitary regulations and significantly shorten the prescribed quarantine periods to simplify and speed up the international movement of horses for trade and sporting purposes.

# Nucleic acid sequence alignments

This appendix contains the aligned sequences used in the phylogenetic study described in Chapter 4. The alignments were carried out with the program Clustal X (Thompson et al., 1999) and contains the AHSV 7 and AHSV 3 reference strain nucleotide sequence data.


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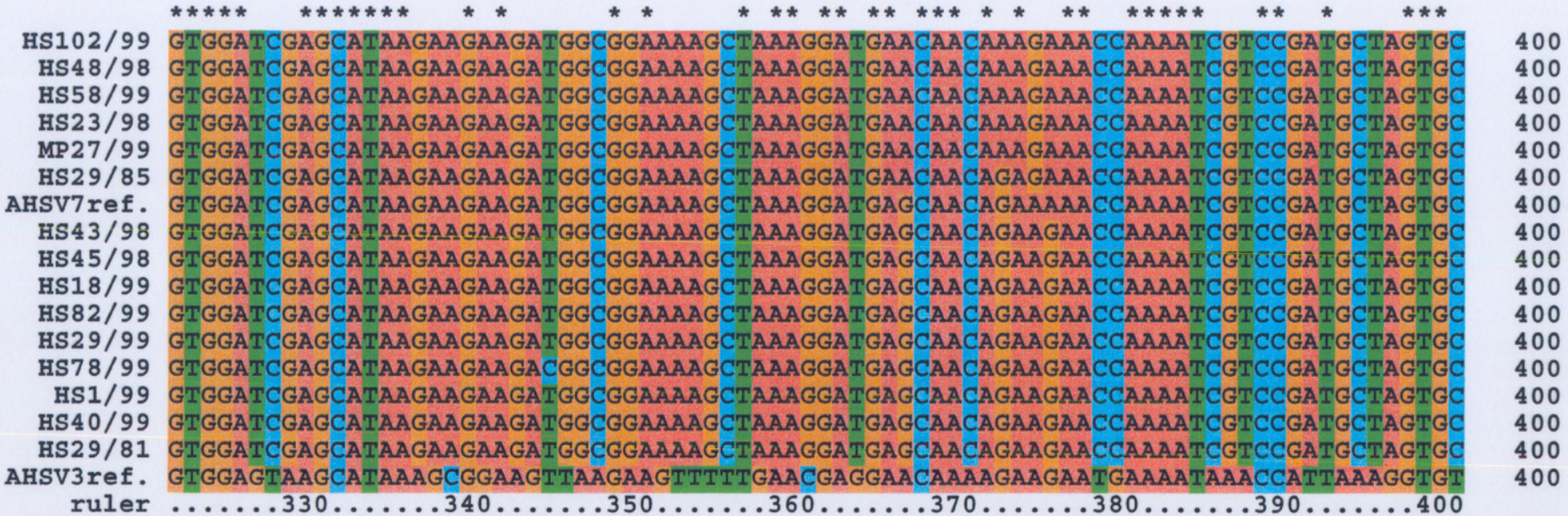
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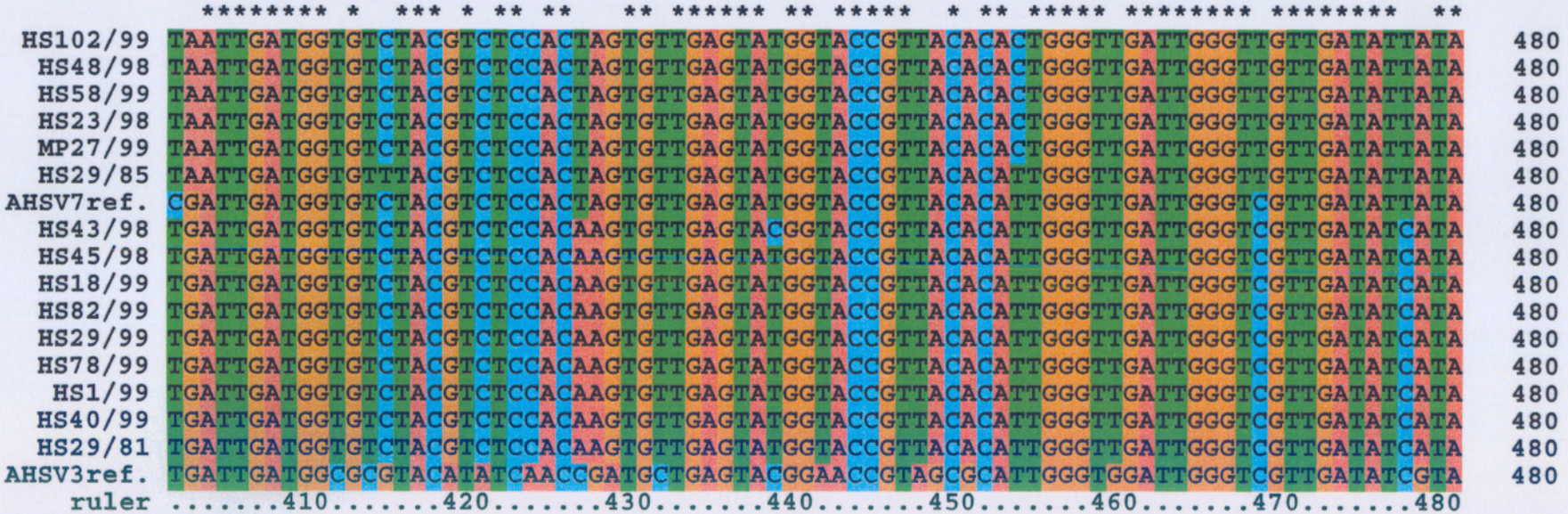
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HS29/81 ATGACAACGCAGGTTGAAAGAATGATAAAAGAGTATGATTTTAAACGATTGGAGAGAAAACAGCTAATTAGTGGTATGAA 560
AHSV3ref. ATGATGACTCAAGTTAGCCGCATGATAAAAGAATATAATTTTATTAGATTAAAAAAGGATCAGCTAATCAGCGGTATGAA 560
ruler .....490.....500.....510.....520.....530.....540.....550.....560
HS102/99 TAAACTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTATGGAGATGCAATTGAGG 640
HS48/98 TAAACTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTATGGAGATGCAATTGAGG 640
HS58/99 TAAACTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTATGGAGATGCAATTGAGG 640
HS23/98 TAAACTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTATGGAGATGCAATTGAGG 640
MP27/99 TAAACTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTATGGAGATGCAATTGAGG 640
HS29/85 CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTATGGAGATGCAATCGAGG 640
AHSV7ref. CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTGTACGATTATTATGGAGATGCGATCGAGG 640
HS43/98 CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTACGGAGATGCAATCGAGG 640
HS45/98 CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTGTTACGGAGATGCAATCGAGG 640
HS18/99 CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTACGGAGATGCAATCGAGG 640
HS82/99 CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTACGGAGATGCAATCGAGG 640
HS29/99 CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTACGGAGATGCAATCGAGG 640
HS78/99 CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTACGGAGATGCAATCGAGG 640
HS1/99 CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTACGGAGATGCAATCGAGG 640
HS40/99 CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATTATACGATTATTACGGAGATGCAATCGAGG 640
HS29/81 CAAGCTCGAGGATGGAGTTAAATGTTATGCTTATTGCTTGATTTTGGCATCATACGATTATTATGGGGATGCAATCGAGG 640
AHSV3ref. TAAGTTGGAGGATGGGGTAAAATGCTACGCGTATTGTTTGATCCTCGCACTGTACGATTTTCATGGGCGTGACGTAGACG 640
ruler .....570.....580.....590.....600.....610.....620.....630.....640


HS102/99 GTTTCGCGCAAGGAACGCGTGCGGGTTCGATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTTCGTTCAGACGTAGCG 720
HS48/98 GTTTCGCGCAAGGAACGCGTGCGGGTTCGATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTTCGTTCAGACGTAGCG 720
HS58/99 GTTTCGCGCAAGGAACGCGTGCGGGTTCGATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTTCGTTCAGACGTAGCG 720
HS23/98 GTTTCGCGCAAGGAACGCGTGCGGGTTCGATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTTCGTTCAGACGTAGCG 720
MP27/99 GTTTCGCGCAAGGAACGCGTGCGGGTTCGATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTTCGTTCAGACGTAGCG 720
HS29/85 GTTTCGCGCAAGGAACGCGCGCGGGTTCGATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTTCGTTCAGACGTAGCG 720
AHSV7ref. GTTTCGCGCAAGGAACGCGTGCGGGTTCGATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAGACGTAGCG 720
HS43/98 GTTTCGCGCAAGGAACGCGTGCGGGTTCAATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAGATGTAGCG 720
HS45/98 GTTTCGCGCAAGGAACGCGTGCGGGTTCAATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAGATGTAGCG 720
HS18/99 GTTTCGCGCAAGGAACGCGTGCGGGTTCAATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAGATGTAGCG 720
HS82/99 GTTTCGCGCAAGGAACGCGTGCGGGTTCAATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAGATGTAGCG 720
HS29/99 GTTTCGCGCAAGGAACGCGTGCGGGTTCAATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAGATGTAGCG 720
HS78/99 GTTTCGCGCAAGGAACGCGTGCGGGTTCAATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAGATGTAGCG 720
HS1/99 GTTTCGCGCAAGGAACGCGTGCGGGTTCAATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAGATGTAGCG 720
HS40/99 GTTTCGCGCAAGGAACGCGTGCGGGTTCAATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAGATGTAGCG 720
HS29/81 GTTTCGCGCAAGGAACGCGTGCGGGTTCGATTGTCGAGACAATCTCCCAGATGTTCCCTGAGTTCCGTTCAGATGTAGCG 720
AHSV3ref. GCTTTGCGCAGGGGACGAGAACCGCAGCTATTGTTGAGACCGTCGCGCGAATGTTTCCTGATTTTCGCTCTGAGGTTTCG 720
ruler .....650.....660.....670.....680.....690.....700.....710.....720
HS102/99 GAAAAGTTTGGTATTAAATTGACTATAAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS48/98 GAAAAGTTTGGTATTAAATTGACTATAAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS58/99 GAAAAGTTTGGTATTAAATTGACTATAAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS23/98 GAAAAGTTTGGTATTAAATTGACTATAAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
MP27/99 GAAAAGTTTGGTATTAAATTGGCTATAAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS29/85 GAAAAGTTTGGTATTAAACTGACTATAAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
AHSV7ref. GAAAAGTTTGGTATTAAACTGACTATAAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS43/98 GAAAAGTTTGGTATTAAATTAACTATTAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS45/98 GAAAAGTTTGGTATTAAATTAACTATTAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS18/99 GAAAAGTTTGGTATTAAATTAACTATTAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS82/99 GAAAAGTTTGGTATTAAATTAACTATTAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS29/99 GAAAAGTTTGGTATTAAATTAACTATTAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS78/99 GAAAAGTTTGGTATTAAATTAACTATTAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS1/99 GAAAAGTTTGGTATTAAATTAACTATTAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS40/99 GAAAAGTTTGGTATTAAATTAACTATTAAGGACGAATCAGAAGAGTTGTTCGTTCAAAAAGATATG 786
HS29/81 GAAAAGTTTGGTATTAAATTAACTATAAAGGACGAATCAGAAGAGTTGTTTGTTCAAAAAGATATG 786
AHSV3ref. GAAAAATTCGGTATTGATTTAGCGGTGTCAGAGGAATCAGATGAACTATTCGTAAAGAAGACGATG 786
ruler .....730.....740.....750.....760.....770.....780.....

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