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# AFRICAN HORSE SICKNESS OUTBREAK INVESTIGATION AND DISEASE SURVEILLANCE USING MOLECULAR TECHNIQUES

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A thesis submitted to the Faculty of Veterinary Science of the University of Pretoria in partial fulfilment of the requirements for the degree of

**Philosophiae Doctor (Veterinary Science)**

August 2016

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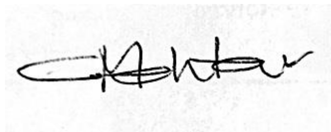

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# ABBREVIATIONS

| | |
|---|---|
| AGID | agar gel immunodiffusion |
| AHS | African horse sickness |
| AHSV | African horse sickness virus |
| BTV | bluetongue virus |
| BTV | bluetongue virus |
| CA | controlled area |
| cDNA | complimentary deoxyribonucleic acid |
| CFR | case fatality rate |
| CFT | compliment fixation test |
| CPE | cytopathic effect |
| Cq | quantification cycle |
| DAFF | Department of agriculture, forestry and fisheries |
| ddNTP | dideoxynucleotides triphosphates |
| DIVA | differentiation of infected from vaccinated animals |
| dsRNA | double-stranded ribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| EEV | equine encephalosis virus |
| ERC | Equine Research Centre |
| EU | European Union |
| FZ | free zone |
| GS | group specific |
| Hgb | Haemoglobin |
| hn | hemi-nested |
| Ht | Haematocrit |
| iELISA | indirect enzyme-linked immunosorbent assay |
| IFA | indirect fluorescent antibody |
| IZ | infected zone |
| LAV | live attenuated vaccine |
| Mab | maternal antibody |
| ML | maximum likelihood |
| NGS | next generation sequencing |
| NS | non-structural protein |
| nt | nucleotide |
| OBP | Onderstepoort Biological Products |



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| | |
|---|---|
| OBP comb1 | combination 1 of the AHS vaccine (serotypes 1, 3 and 4) |
| OBP comb2 | combination 2 of the AHS vaccine (serotypes 2, 6, 7 and 8) |
| OD | optical density |
| OIE | World Organisation for Animal Health (Office International des Epizooties) |
| OVI | Onderstepoort Veterinary Institute |
| PCR | polymerase chain reaction |
| PI | plaque inhibition |
| PP | percentage positive |
| PZ | protection zone |
| RBC | red blood cell |
| RCC | Red cell count |
| RT-qPCR | reverse transcriptase quantitative PCR |
| SA | South African |
| SNT | serum neutralisation test |
| SNV | single nucleotide variant |
| SV | state veterinarian |
| SZ | surveillance zone |
| TS | type specific |
| VI | virus isolation |
| VP | viral protein |
| WCP | Western Cape Province |
| WNV | West Nile virus |



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# TABLE OF CONTENTS

**DECLARATION** .......... **I**

**ACKNOWLEDGEMENTS** .......... **II**

**ABBREVIATIONS** .......... **IV**

**TABLE OF CONTENTS** .......... **VI**

**LIST OF FIGURES** .......... **X**

**LIST OF TABLES** .......... **XVI**

**SUMMARY** .......... **1**

**GENERAL INTRODUCTION** .......... **5**

**CHAPTER 1** .......... **8**

LITERATURE REVIEW .......... 8

*1.1 Overview* .......... *8*

*1.2 Pathogenesis* .......... *10*

*1.3 Clinical signs* .......... *11*

*1.4 Macroscopic pathology* .......... *12*

*1.5 Clinical pathology* .......... *12*

*1.6 Control* .......... *13*

*1.7 AHSV Structure* .......... *16*

*1.8 Viral reassortments and reversion to virulence* .......... *18*

*1.9 Diagnostic Techniques* .......... *19*

*1.10 AHS Controlled Area of South Africa* .......... *28*



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**CHAPTER 2 .......... 32**

THE 2011 OUTBREAK OF AFRICAN HORSE SICKNESS IN THE AFRICAN HORSE SICKNESS CONTROLLED AREA IN SOUTH AFRICA .......... 32

*2.1 Abstract .......... 32*

*2.2 Introduction .......... 32*

*2.3 Materials and methods .......... 33*

*2.4 Observations .......... 38*

*2.5 Discussion .......... 41*

*2.6 Conclusion .......... 45*

**CHAPTER 3 .......... 46**

DEVELOPMENT OF THREE TRIPLEX REAL-TIME REVERSE TRANSCRIPTION PCR ASSAYS FOR THE QUALITATIVE MOLECULAR TYPING OF THE NINE TYPES OF AFRICAN HORSE SICKNESS VIRUS ... 46

*3.1 Abstract .......... 46*

*3.2 Introduction .......... 46*

*3.3 Materials and Methods .......... 48*

*3.4 Results .......... 53*

*3.5 Discussion .......... 57*

*3.6 Conclusion .......... 59*

**CHAPTER 4 .......... 60**

EVALUATION OF THE USE OF FOALS FOR ACTIVE SURVEILLANCE IN AN AFRICAN HORSE SICKNESS CONTAINMENT ZONE DURING THE SEASON FOLLOWING AN OUTBREAK. .......... 60

*4.1 Abstract .......... 60*

*4.2 Introduction .......... 60*



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*4.3 Materials and Methods* ........ *63*

*4.4 Results* ........ *66*

*4.5 Discussion* ........ *67*

**CHAPTER 5** ........ **69**

DYNAMICS OF AFRICAN HORSE SICKNESS VIRUS NUCLEIC ACID AND ANTIBODY IN HORSES FOLLOWING IMMUNISATION WITH A COMMERCIAL POLYVALENT ATTENUATED LIVE VIRUS VACCINE ........ 69

*5.1 Abstract* ........ *69*

*5.2 Introduction* ........ *69*

*5.3 Materials and Methods* ........ *70*

*5.4 Results* ........ *74*

*5.5 Discussion* ........ *85*

*5.6 Conclusion* ........ *87*

**CHAPTER 6** ........ **88**

AFRICAN HORSE SICKNESS CAUSED BY GENOME REASSORTMENT AND REVERSION TO VIRULENCE OF LIVE, ATTENUATED VACCINE VIRUSES, SOUTH AFRICA, 2004 - 2014 ........ 88

*6.1 Abstract* ........ *88*

*6.2 Introduction* ........ *88*

*6.3 Materials and Methods* ........ *91*

*6.4 Results* ........ *94*

*6.5 Discussion* ........ *113*

**GENERAL CONCLUSIONS** ........ **116**

**REFERENCES** ........ **118**



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**APPENDICES ........ 141**

APPENDIX A ........ 141

APPENDIX B ........ 147

APPENDIX C ........ 151

APPENDIX D ........ 155

APPENDIX E ........ 156

APPENDIX F ........ 157

APPENDIX G ........ 158



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# LIST OF FIGURES

Figure 1-1: A map of the zones within the African horse sickness (AHS) Controlled Area of South Africa. ........ 10

Figure 1-2: A diagrammatic representation of the structure of the African horse sickness viral particle, showing the positioning of the seven structural viral proteins (VP1-7) and the segmented RNA genome (after Wilson et al. 2009). ........ 16

Figure 1-3: The plaque inhibition neutralization test: wells numbered 1-3 are the controls. Wells numbered 1 & 2 show viral plaque growth (multifocal pale areas) and well 3 is the negative control with no inoculated virus on a monolayer of Vero cells. Wells numbered 4-6 show monolayers with fish-spine beads (three beads per well) with antiserum to a different type in each bead. Well 5 shows a zone of inhibition around the right most bead where antibody neutralisation of AHSV has occurred. ........ 21

Figure 2-1: A map indicating the extent of the AHS outbreak in the Western Cape. Section A shows the geographical extent of South Africa with the Provincial boundaries as well as the extent frame of section B (labelled as such). Section B shows two of the AHS control zones within the Western Cape Province, namely the Protection zone which is an area of compulsory vaccination for domestic equids against AHS; and the Surveillance zone which is where vaccination is only performed after permission from the Provincial Director of Veterinary Services is obtained. Also in Section B is the extent of the Malmesbury magisterial district, the boundaries of which were used to delineate the movement restriction zone implemented during the outbreak. Section C shows the extent of the outbreak by individual confirmed cases (n=73) within the Malmesbury district as well as the extent of the Mamre town limits which was the epicentre of the outbreak and where the majority (n= 57) of cases occurred........ 34

Figure 2-2: The epidemic curve (dashed line) of the outbreak depicting only AHS confirmed cases (n=73). The peak of cases occurred during the fourth week (including week zero) of the outbreak with 22 cases occurring. The curve drops off sharply with a small secondary peak during the eighth week of the outbreak. The vaccinations performed as a result of the outbreak (private veterinarian vaccinations not included; totals per week shown as bars with the totals on the right axis) show that the peak vaccination occurred in the week before the peak in confirmed cases. ........ 40



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Figure 2-3: A neighbour-joining phylogenetic tree showed that the outbreak isolate (E14311 VP2 0001 1003) grouped with other AHSV type one isolates. ........................................... 41

Figure 3-1: A map of Namibia and South Africa with its provinces. The labels on the map refer to Namibia (NAM) and the nine provinces of South Africa, namely Eastern Cape (EC), Free State (FS), Gauteng (GP), KwaZulu-Natal (KZN), Limpopo (LP), Mpumalanga (MP), North West (NW),Northern Cape (NC) and the Western Cape Province (WC). The total number of positive AHSV TS RT-qPCR samples per type for samples collected between 1 January 2011 and 31 May 2012 is depicted for each area. ............................................... 55

Figure 4-1: A map showing the extent of the Mamre African horse sickness (AHS) outbreak in 2011, as well as the locations of the animals that were sampled in this study. The AHS free, surveillance and protections zones of the AHS controlled area are indicated. ............ 61

Figure 4-2: A map showing the spatial distribution of the Holdings where the foal sentinels where situated (Blue dots) in relation to the confirmed cases of AHS (red dots) and the extent of the outbreak (polygon) during the 2011 Mamre AHS outbreak. Location A) Darling, B) Quarry, C) Skurwekraal, D) Mamre Town and E) Nature Reserve. ................................ 64

Figure 5-1: Group Timelines depicting the vaccination schedule and serological sample collection times for each of the 4 weanlings treatment groups. .......................................... 72

Figure 5-2: Barplots showing the total number of weanlings positive on group specific RT-qPCR over time in each of the 4 weanling groups. Weanling group I initially received African horse sickness live attenuated vaccine combination 1 (OBP comb1) at week 0, and OBP comb2 4 weeks later (OBP comb2 is indicated by **). Weanling group II received OBP comb2 at week 0 and OBP comb1 4 weeks later (comb1 is indicated by *). Weanling group III received OBP comb1 at week 0 and OBP comb2 12 weeks later (**). Weanling group IV received OBP comb2 at week 0 and OBP comb1 12 weeks later (*). The weanling groups that received OBP comb1 initially are indicated by blue bars. Weanling groups that received OBP comb2 initially are indicated by orange bars. ......................................................... 76

Figure 5-3: A box and whisker plot describing the Cq value distributions of the GS RT-qPCR positive weanlings in each of the 4 AHS weanling groups (Groups I to IV). The solid line within the boxes represents the median Cq value. The outlier results are represented by the dots. Weanling group I received African horse sickness live attenuated vaccine combination 1 (OBP comb1) at week 0, and OBP comb2 4 weeks later (comb2 is indicated by **). Weanling group II received OBP comb2 at week 0 and OBP comb1 4 weeks later (comb1 is



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indicated by *). Weanling group III received OBP comb1 at week 0 and OBP comb2 12 weeks later (**). Weanling group IV received OBP comb2 at week 0 and OBP comb1 12 weeks later (*). The weanling groups that received OBP comb1 initially are indicated by blue bars. Weanling groups that received OBP comb2 initially are indicated by orange bars...... 77

Figure 5-4: Stacked Bar plots depicting the number of type specific (TS) RT-qPCR positive weanlings for each of the 9 AHSV types on TS-RT-qPCR in each of the 4 weanling groups over time. Plot a) depicts Weanling group I, b) Weanling group II, c) Weanling group III and d) Weanling group IV. Plots a) and c) depicted in blue shades, received OBP comb1 at week 0 and OBP comb2 at weeks 4 and 12 respectively. Plots b) and d) depicted in orange shades, received OBP comb2 at week 0 and OBP comb1 at weeks 4 and 12 respectively. The stacks indicate the number of weanlings that tested positive on TS RT-qPCR per AHSV type in the first 4 week, between weeks 4 and 8, weeks 8 and 12, and weeks 12 and 16. Once a weanling tested positive for a specific type it was not counted for that type again... 79

Figure 5-5: Serological responses of weanlings following immunisation with a polyvalent AHSV-LAV. Stacked Bar plots depicting the number of weanlings that seroconverted (SNT titre > 10) for each of the 9 AHSV types. Plot a) depicts Weanling group I, b) Weanling group II, c) Weanling group III and d) Weanling group IV. Plots a) and b) depicted in blue shades, received OBP comb1 at week 0 and OBP comb2 at weeks 4 and 12 respectively. Plots c) and d) depicted in orange shades, received OBP comb2 at week 0 and OBP comb1 at weeks 4 and 12 respectively. The stacks indicate the number of weanlings that seroconverted per AHSV type in the first 4 week, between weeks 4 and 8, weeks 8 and 12, and weeks 12 and 16. ........................................................................................ 81

Figure 5-6: Barplots comparing the total number of positive animals on GS RT-qPCR for Weanling group 1 after the weanling vaccinations at week 0 (a) and yearling vaccinations at week 16 (b) and comparing Weanling group 2 after weanling vaccinations at week 0 (c) and yearling vaccinations at week 16 (d). The x-axis represents the number of weeks after the initial vaccine bottle for each group. Weanling group 1 received OBP comb1 at week 0 followed by OBP comb2 four weeks during their weanling vaccinations. Weanling group 2 received OBP comb2 followed by OBP comb1 four weeks later during their weanling vaccinations. Both groups received OBP comb1 followed by OBP comb2 during their yearling vaccinations at week 16. ........................................................................ 83

Figure 6-1: Map showing African horse sickness (AHS) outbreaks included in this study. Map of the AHS controlled area of the Western Cape Province of South Africa showing the spatial distribution of each of the AHS virus type 1 outbreaks that have occurred in this area since



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1997. The AHS free, surveillance and protection zones are also shown. Individual confirmed cases of AHS are indicated by the solid dots. The directional distribution of each outbreak is indicated using standard deviational ellipses. .......... 91

Figure 6-2: Whole genome phylogeny of African horse sickness viruses (AHSV). Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated whole genome nucleotide sequences of viruses from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS CA to the AHSV live, attenuated vaccine and reference viruses. Branches are scaled to represent numbers of inferred nucleotide differences per site with the scale bar at the bottom of the tree indicating genetic distance. Branches supported by full maximum likelihood bootstrap values >70% are indicated. Genotype groups are indicated with brackets and group names to the right of the tree. .......... 96

Figure 6-3: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP1 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. Branches are scaled to represent numbers of inferred nucleotide differences per site with the scale bar at the bottom of the tree indicating genetic distance. Branches supported by full maximum likelihood bootstrap values >70% are indicated. Genotype groups are indicated with brackets and group names to the right of the tree. .......... 97

Figure 6-4: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP2 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details. .......... 98

Figure 6-5: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP3 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details. .......... 99

Figure 6-6: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP4 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details. .......... 100

Figure 6-7: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP5 nucleotide sequences from affected horses in the 2004, 2011 and 2014



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outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details. ........ 101

Figure 6-8: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP6 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details. ........ 102

Figure 6-9: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP7 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details. ........ 103

Figure 6-10: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated NS1 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details. ........ 104

Figure 6-11: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated NS2 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details. ........ 105

Figure 6-12: Maximum-likelihood phylogenetic tree indicating the genetic relationships of concatenated NS3 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details. ........ 106

Figure 6-13: Cladogram and heatmap of vaccine-derived African horse sickness virus reassortants. Cladogram indicating the genetic relationships of concatenated African horse sickness viruses (AHSV) whole genome nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS controlled area with a heatmap summarising the origin of the gene segments for each strain with 1/Lab/ZAF/98/OBP-116, 3/Lab/ZAF/98/OBP-116 and 4/Lab/ZAF/98/OBP-116 vaccine derived strains represented by green, blue and red blocks, respectively. Grey blocks indicate that segment could be derived from either 1/Lab/ZAF/98/OBP-116 or 3/Lab/ZAF/98/OBP-116. Branches supported by full maximum likelihood bootstrap values >70% are indicated. Genotype groups are indicated with brackets and group names to the right of the tree. ........ 107



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Figure 6-14: Statistical evidence of reassortment within the genomes of AHSV field isolates. A Hidden Markov model based approach (called BURT-HMM) was used to classify individual nucleotides within each of the 10 segments of individual AHSV isolates into three different categories: 1/Lab/ZAF/98/OBP-116-like (in green), 3/Lab/ZAF/98/OBP-116-like (in blue) and 4/Lab/ZAF/98/OBP-116-like (in red). Probability supports for these classifications yielded by the BURT-HMM with the highest likelihood are plotted along the genome with the positions of segment boundaries given in the diagram above the plots. The phylogenetic clusterings that are implied by differently coloured segments in these plots are indicated below the plots. Note that the segment indicated in grey could not be convincingly classified because it very closely resembles both 1/Lab/ZAF/98/OBP-116 and 3/Lab/ZAF/98/OBP-116. .................. 109



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# LIST OF TABLES

Table 2-1: The outbreak case definition codes categorised according to the clinical signs and laboratory results of each case, and the number of cases in each category. ...................... 36

Table 2-2: Various epidemiological variables measured during the outbreak. Equines evaluated include all equines which were surveyed using RT-qPCR testing by the Equine Research Centre (UP). Population at risk is based on the census performed in the outbreak area by the veterinary services of the Western Cape. 95% confidence intervals have been included in brackets where applicable. ................................................................ 38

Table 3-1: Information on the origin of the African horse sickness virus reference strains (2014); showing the name, isolate name and country of isolation with passage history and GenBank accession numbers for genes encoding VP2 of each virus. ............................... 49

Table 3-2: African horse sickness virus type specific primers and minor groove binding (MGB) probes for the three triplex assays. ......................................................... 51

Table 3-3: Evaluation of two sets of reference strains of the 9 types of African horse sickness virus using group specific (AHSV RT-qPCR) and type specific (AHSV TS RT-qPCR) assays. ............................................................................................................ 53

Table 3-4: African horse sickness virus types identified in equine blood samples by type specific AHSV TS RT-qPCR from cases of African horse sickness confirmed by group specific AHSV RT-qPCR. ....................................................................................... 54

Table 3-5: A summary of the total number of samples positive by TS specific RT-qPCR and virus isolation. The tables indicate ST RT-qPCR positive cases and viral isolation positive cases for each TS of African horse sickness in each province of South Africa and Namibia (see Figure 3-1) during 2011 and 2012. ........................................................... 56

Table 3-6: Results of Group Specific (AHSV RT-qPCR) and type specific (AHSV TS RT-qPCR) assays performed on archived blood samples from clinical and subclinical cases of African horse sickness. ..................................................................................... 57

Table 4-1: A table showing the location name, identification (name and microchip number) and month of birth (MOB) of the foals used in this study. ................................................ 65



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Table 4-2: Summary of results of the RT-qPCR assays performed on EDTA blood samples from each foal each month and of iELISA assays performed on serum samples from each foal at the beginning and end of the study. For the RT-qPCR, a Cq > 37 was considered negative. For the iELISA, a percentage positive value > 10 was considered positive. NS indicates that no samples were collected that month. The age in months (m) of each foal is given as the age at start of study in January 2012. ........................................................ 66

Table 5-1: African horse sickness virus (AHSV) type specific primers and minor groove binding (MGB) probe for AHSV type 7 including the vaccine strain with the truncated gene for VP2. ........................................................................................................ 73

Table 5-2: A comparison between the different weanling treatment groups using the Wilcoxon Rank-Sum test. The table shows the *p-values* calculated for comparisons between each of the treatment groups. *A *p-value* < 0.05 indicates a significant difference. ............. 79

Table 5-3: GS RT-qPCR results and TS RT-qPCR results for Onderstepoort Biological Products polyvalent OBP comb1 and comb2 ..................................................................... 80

Table 5-4: A summary of the Group Specific RT-qPCR (GS) and Type Specific RT-qPCR (TS) results over time for each of the yearlings in Weanling group I and II. The Cq value for each of the GS results is given, and the TS result indicating which AHS type was obtained is given below each corresponding positive GS result. N indicates that the Cq value for the GS PCR was > 37. The Minimum Cq per week is given as well as the total number of positive yearlings on GS PCR per week. ND means no AHSV type could be detected. The column highlighted in red indicates the week when OBP comb1 was given (types 1, 3 and 4) and the column highlighted in green indicates the week OBP comb2 (types 2, 6, 7 and 8) was given. ........................................................................................................................ 82

Table 5-5: A summary of the Group Specific RT-qPCR (GS) results over time for each of the mares. Group A consisted of mares that had received 3 to 5 AHS vaccinations in their life (n=7), and Group B, mares that have received 6 of more AHS vaccinations (n=15). N indicates that the Cq value for the GS PCR was > 37. The * indicates that the sample was not tested. The column highlighted in red indicates the week when OBP comb1 was given (types 1, 3 and 4) and the column highlighted in green indicates the week OBP comb2 (types 2, 6, 7 and 8) was given. The number of vaccination previously received for each of the mares is given. ........................................................................................ 84



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Table 6-1: Summary of results of best fit substitution models obtained using PhyML-SMS (Smart Model Selection) with the Akaike Information Criterion for the concatenated alignments of the complete AHSV genomes and individual AHSV gene segments of the strains included in this study. .......................................................................................... 94

Table 6-2: RAMI indices describing the microdiverse clusters identified in AHSV1 isolates from the 2004, 2011 and 2014 outbreaks of AHS in the Western Cape Province of South Africa. ......................................................................................................................... 95

Table 6-3: Summary statistics (P values) of tests for reassortment of gene segments of AHSV using seven methods incorporated within RDP4.................................................. 108

Table 6-4: Summary of single nucleotide variations (SNVs) observed in NS1 of 1/Lab/ZAF/98/OBP-116 and 3/Lab/ZAF/98/OBP-116 vaccine derived strains and strains isolated during 2014 AHS outbreaks in Porterville and Robertson. [a] Position given as nucleotide and (amino acid). [b] SNV's given as nucleotide and (amino acid)...................... 110

Table 6-5: A summary of the attenuation-associated non-synonymous single nucleotide variants (nsSNVs) of the consensus sequences of the genome segments of the African horse sickness type 1 (AHSV-1) Laboratory Reference Strain, 1/Lab/ZAF/98/OBP-116 vaccine-derived virus and AHSV-1 viruses from the 2004 Stellenbosch, 2011 Mamre, 2014 Porterville and 2014 Robertson AHS outbreaks. ............................................................ 111

Table 6-6: Summary of the epidemiological parameters for each of the outbreaks involving AHSV-1 in the AHS controlled area of the Western Cape Province of South Africa in 2004, 2011 and 2014. ........................................................................................................ 113



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# SUMMARY

By

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Supervisor: Prof AJ Guthrie

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Department: Veterinary Tropical Diseases

Degree: PhD

African horse sickness (AHS) is a life-threatening disease of equids caused by African horse sickness virus (AHSV), a member of the genus *Orbivirus* in the family *Reoviridae*. The virus is transmitted by midges (*Culicoides* spp.) and the disease is most prevalent during the time of year, and in areas where vector *Culicoides spp*. are most abundant, namely in late summer in the summer rainfall areas of endemic regions. The disease is of importance to health and international trade in horses worldwide. Effective surveillance is critical in order to establish transparent criteria for animal trade from a country or region where AHS occurs.

**The 2011 outbreak of African horse sickness in the African horse sickness controlled area in South Africa:** An outbreak of AHS caused by AHSV type one (AHSV1) occurred in the surveillance zone of the AHS controlled area of the Western Cape during the summer of 2011. The epicentre of the outbreak was the town of Mamre in the magisterial district of Malmesbury, and the outbreak was confined to a defined containment zone within this area through movement control of all equids and a blanket vaccination campaign. A total of 73 confirmed cases of AHS were reported during this outbreak, which included four subclinical cases confirmed by virus isolation (VI). The estimated morbidity rate for the outbreak was 16% with an estimated mortality rate of 14% and a case fatality rate of 88% based on the figures above. Outbreak disease surveillance relied on agent identification using AHSV group specific reverse transcriptase quantitative polymerase chain reaction (GS RT-qPCR) based assays, which was novel for an AHS outbreak in South Africa. The source of this outbreak was not confirmed at the time, but was believed to be associated with an illegal



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movement of an infected animal into the Mamre area. A detailed description of the outbreak is given in Chapter 2, and the outbreak provided an opportunity to assess decision making in future AHS outbreaks in the AHS controlled area of South Africa and in countries where AHS is an exotic or emerging disease. This outbreak further highlighted deficiencies and complications of available AHSV diagnostic testing and surveillance methods, and the need for further refinement of these assays and strategies.

**Development of three triplex real-time reverse transcription PCR assays for the qualitative molecular typing of the nine types of African horse sickness virus:** The typing of the specific AHSV involved in the Mamre outbreak was initially done by partial, direct sequencing of the S10 gene (encoding the non-structural protein NS3) and the L2 gene (encoding the type-specific outer capsid protein VP2) which confirmed the virus to be AHSV1. This process is time consuming and it became evident that a faster alternative was needed. This led to the development of type specific RT-qPCR (TS RT-qPCR) assays to supplement the GS RT-qPCR assay that had already been developed, characterized and validated. Blood samples collected during routine diagnostic investigations from South African horses with clinical signs suggestive of AHS were subjected to analysis with the GS RT-qPCR assay and VI with subsequent serotyping by plaque inhibition (PI) assays using AHSV type-specific antisera. Blood samples that tested positive by AHSV GS RT-qPCR were then selected for analysis using AHSV TS RT-qPCR assays. The TS RT-qPCR assays were evaluated using both historic stocks of the South African reference strains of each of the 9 AHSV types, as well as recently derived stocks of these same viruses. Of the 503 horse blood samples tested, 156 were positive by both AHSV GS RT-qPCR and VI assays, whereas 135 samples that were VI negative were positive by AHSV GS RT-qPCR assay. The virus isolates made from the various blood samples included all 9 AHSV types, and there was 100% agreement between the results of conventional serotyping of individual virus isolates by PI assay and AHSV TS RT-qPCR typing results. Results of this study confirmed that the AHSV TS RT-qPCR assays for the identification of individual AHSV types are applicable and practicable and therefore are potentially highly useful and appropriate for virus typing in AHS outbreak situations in endemic or sporadic incursion areas, which can be crucial in determining appropriate and timely vaccination and control strategies.

**Evaluation of the use of foals for active surveillance in an AHS containment zone during the season following an AHS outbreak:** In order to further evaluate the AHS status of horses in the Mamre area after the outbreak of 2011, a targeted surveillance strategy was developed. Serial serum and whole blood samples were collected on a monthly basis from January to June, 2012 from foals (identified by microchip) that were born in the Mamre



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district after the end of the outbreak. Sera were evaluated using traditional serological methods and the results were compared to the results obtained using the newly developed molecular assays for virus detection and identification. This study confirmed that AHSV was eradicated in the Mamre area after the outbreak and, therefore, that the control measures implemented in the area by the State Veterinary Authorities were effective.

**Characterization of the dynamics of African horse sickness virus in horses by assessing the RNAaemia and serological responses following immunisation with a commercial polyvalent live attenuated vaccine:** As was shown in the 2011 Mamre outbreak, detection of AHSV during outbreaks has become more rapid and efficient with the recent development of quantitative GS RT-qPCR assays to detect AHSV nucleic acid. Use of this assay together with the TS RT-qPCR assays described in Chapter 3, will not only expedite diagnosis of AHS but also facilitate further evaluation of the dynamics of AHSV infection in the equine host. A potential limitation to the application of these assays is that they detect viral nucleic acid originating from any AHSV live attenuated vaccine (AHSV-LAV), which is the vaccine type routinely administered to horses in South Africa. A study was, therefore, designed to characterize the dynamics and duration of the RNAaemia as compared to the serological responses of horses following vaccination with a commercial AHSV-LAV, using GS and TS RT-qPCR assays and serum neutralisation tests. This study provided baseline data on the GS and TS nucleic acid dynamics in weanling foals vaccinated for the first time, yearlings vaccinated for a second time and adult mares following a booster to multiple previous vaccinations. These data are fundamental to interpreting results of AHSV GS RT-qPCR testing of vaccinated horses within an area where virological surveillance is being applied.

**African horse sickness caused by genome reassortment and reversion to virulence of live, attenuated vaccine viruses, South Africa, 2004 - 2014:** In 2014 a further outbreak of AHS caused by AHSV1 occurred in the Porterville area of the AHS protection zone (PZ), spreading into the Wellington area in the AHS surveillance zone (SZ). Further involvement of the Robertson area (AHS PZ) subsequently also occurred. The case fatality rate was much lower than that of the Mamre outbreak. The clinical signs in infected horses were also generally milder in the 2014 outbreak, as compared to the 2011 outbreak. Whole genome sequencing of samples from the Porterville outbreak confirmed that causative virus was a recombination (reassortant) of AHSV types 1 and 4, with genes derived from the relevant vaccine strains contained in OBP comb1 of the commercial polyvalent AHSV-LAV used in South Africa. This led to further analysis of 39 AHSV strains from field cases of AHS that originated from outbreaks within the controlled area, which confirmed reversion to virulence



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of AHSV type 1 vaccine in two outbreaks (2004 and 2011) and multiple reassortment events in two outbreaks (2004 and 2014) with genes derived from all three AHSV vaccine strains (types 1, 3 and 4). This study provided a molecular and epidemiological comparison of the five unique AHSV type 1 outbreaks in the AHS controlled area. It was shown that all the outbreaks in the AHS controlled area attributed to AHSV type 1 since the inception of the area in 1997, have been due either to reversion to virulence of the AHSV type 1 vaccine strain, or recombination of AHSV type 1 vaccine strain with one or both of the other vaccine strains in OBP comb1 of the commercial AHSV-LAV.



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## GENERAL INTRODUCTION

African horse sickness (AHS) is a non-contagious disease of equids characterized by vascular injury that can result in four forms of disease; the pulmonary, cardiac, mixed or horse sickness fever forms *(62)*. AHS is an OIE-listed disease as it causes high mortality rates and has the potential for rapid spread *(137)*. African horse sickness virus (AHSV) is endemic to sub-Saharan Africa with regular outbreaks occurring in southern Africa *(19, 23)*. The disease was first noticed after the introduction of horses (which were not indigenous to southern Africa) into South Africa from Europe and the Far East *(74)*. Although deaths occur every year in southern Africa from AHS, major epidemics tend to occur approximately every 20 years and are associated with above average rainfall patterns *(17, 65, 113)* and may also be associated with the occurrence of the El Niño weather patterns *(19)*. One of the most devastating recorded outbreaks in southern Africa occurred in 1854/55, when it is estimated that up to 40% of the equine population of the Cape colony died *(17, 74, 83)*.

AHS has spread outside of Africa in the past. An outbreak in the Middle East and South-West Asia in 1960 led to the death of in excess of 300 000 horses *(65, 83)*. This outbreak introduced the “Horse Sickness Ban”, which stopped direct export of horses from Africa. Europe has also suffered outbreaks (Spain 1966 and 1987-90; Portugal 1989). The Spanish outbreak in 1966 was thought to have spread into Spain from Morocco *(73)* possibly from AHSV infected midges carried into Spain by large gusts of wind or by boat *(140)*. The 1987-90 outbreak in the Iberian Peninsula started in Spain (1987) and was linked to zebra that were imported from Namibia. This outbreak subsequently spread to Portugal and Morocco and the causative virus is thought to have “overwintered” in the Southern regions of Spain before it disappeared entirely in 1990 *(95, 139)*.

The recent (since 1998) emergence of multiple types of bluetongue virus (BTV) in southern Europe has created a great deal of concern. The invasion of BTV into Mediterranean Europe was followed by an outbreak in Northern Europe cause by type 8 in 2006 *(101)*. Changes in climate are thought to be an important contributing factor to the expansion of the global range of BTV; however other factors such as possible iatrogenic introduction of new strains in the form of live attenuated vaccines (LAV) and the increase in movement of virus-infected hosts and vectors may also contribute *(98)*. Considering the similarities in epidemiology of bluetongue and AHS it is feared that an outbreak of AHS in Europe could follow *(99, 136)* and could lead to large scale losses if the disease were to become established in Europe. For this reason, control measures have been put in place by the European Union governing the movement of equids *(16)*.



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The area around the Cape of Good Hope in South Africa has historically been free from AHS, with sporadic outbreaks thought to have been due to the introduction of AHSV-positive horses from other provinces. In an effort to maintain this area as an AHS free zone for export purposes, and in order to comply with the EU regulations, movement control of equids into the area has been implemented by the South African State Veterinary Authorities since 1997 *(16, 64)*. The Western Cape Province (WCP) is currently divided into 3 zones; the Metropolitan Cape Town AHS free zone, the AHS surveillance zone, and the AHS protection zone *(157)* collectively known as the AHS controlled area (CA). Horses moving into the AHS CA in the WCP from other provinces must have been vaccinated with the Onderstepoort Biological Products (OBP) polyvalent live attenuated vaccine (LAV), a veterinarian must certify them healthy prior to movement, and a movement permit supplied by the State Veterinarian of their area must accompany them. Even with the current movement controls in place, AHS outbreaks have occurred in the AHS CA, namely in 1999, 2004, 2011, 2013 and 2014 in the surveillance zone and 2006 and 2014 in the protection zone. The outbreak in the AHS protection zone in 2006 *(135)* most likely resulted from the movement of a subclinically AHSV-infected horse into the area *(176)*. The occurrence of subclinical AHSV infection of horses in an endemic area has been described previously *(176)*.

The outbreak in Mamre in 2011 was the first outbreak where surveillance was done with molecular techniques (PCR) on a large scale. Samples were taken not only from clinical cases (as was done in the 2004 outbreak) but from in-contact horses as well, leading to the first documentation of subclinical cases of AHS in unvaccinated individuals. The outbreak was confirmed to have been caused by AHSV type 1 by partial sequencing of the S10 and L2 genes. Vaccination in response to the outbreak was carried out on a large scale in the outbreak containment area, and OBP comb1 (containing AHSV types 1, 3 and 4) of the AHSV-LAV was used due to the AHSV-1 involvement. This outbreak highlighted the need for diagnostic assays able to quickly and accurately identify the AHSV type. Furthermore the effect of vaccination of horses with the AHSV-LAV on interpretation of results, and the possibility of vaccine virus viraemia, were emphasized.

In the 2014 outbreak, the first case of AHS was identified in the Porterville area on the 5$^{th}$ March, and was confirmed by GS RT-qPCR on the 7$^{th}$ March. The symptoms were very mild, with only pyrexia and mild supraorbital fossae swelling being evident, and so it was initially attributed to equine encephalosis virus (EEV) infection. Due to the mild symptoms and the fact that the equine population was fairly stable with little movement into and out of the area, and the fact that vaccination had been done on the index farm as well as the neighbouring farm in November and December 2013, transmission of the AHSV-LAV was suspected to be



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responsible for the outbreak. This suspicion was later confirmed by whole genome sequencing that confirmed multiple reassortment events between vaccine strains of AHSV types 1, 3 and 4. Further investigation of the other outbreaks attributed to AHSV type 1 in the AHS controlled area revealed a reversion to virulence of type 1 vaccine strain in 2004 and 2011, and reassortment events of the types found in OBP comb1 in 2004 and 2014.



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# CHAPTER 1

# Literature Review

## 1.1 Overview

AHS is an infectious, non-contagious, arthropod-borne disease caused by AHSV, a segmented, double-stranded ribonucleic acid (dsRNA) virus (genus *Orbivirus* in the family *Reoviridae*) *(30, 114)*. Horses are the equids most susceptible to AHS, with AHSV infection often leading to high mortalities of up to 90% *(62)*. Donkeys and mules are also susceptible, but generally only develop a mild form of the disease *(40, 47, 68)*. Zebras are highly resistant to the disease *(13, 43, 62)*. It is speculated that a continuous transmission cycle between zebras and the *Culicoides* midges occurs in the Kruger National Park *(15)*, where zebra serve as a critical reservoir for AHSV *(13)*. Large donkey populations could play a similar role under the right climatic conditions *(68)*. AHSV also infects large carnivores via oral routes and dogs as well, although the epidemiologic significance of these species remains uncertain *(4, 165)*.

There are nine antigenically distinct types of AHSV, with serologic cross-reactivity (neutralisation) between types 1 and 2; 3 and 7; 5 and 8; and 6 and 9 *(83, 109)*. The virus is transmitted by *Culicoides* spp., namely *Culicoides imicola* and *C. bolitinos*, and is most prevalent during the time of year, and in areas where, the *Culicoides* spp. are most abundant, namely in late summer in the summer rainfall areas of southern Africa *(18, 36, 112)*. The adult *Culicoides* become infected by taking blood meals from viraemic animals. The incubation period for the virus in the midges is approximately eight days, after which the virus localises in the salivary glands and is transmitted to the next host when the midge takes a blood meal *(95)*. *C. sonorensis (previously named C. varipennis) (76)* has also been shown to be capable of transmitting the disease under experimental conditions, but is prevalent in the USA and not in southern Africa *(21)*. Early and heavy rains followed by warm, dry weather favour the occurrence of AHS and therefore the first cases of AHS in southern Africa usually occur in late February, with the majority of cases occurring in March and April. Whereas many *Culicoides* spp breed in damp soil rich in organic matter, *C. bolitinos* breeds in bovine dung, and is therefore not as dependant on annual rainfall and soil-type *(168)*. Horses kept on open pasture, in low-lying wet areas, and not stabled at night are the most at risk of contracting the disease. The *Culicoides* species responsible for AHS transmission are most active from sunset to sunrise, and *C. imicola* does not readily enter buildings *(14, 30)*. After the first frosts the disease disappears abruptly *(30)*. Transovarial



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transmission of the virus in vectors has not been demonstrated *(95)*. However, the presence of blood fed *C. imicola* females have been found throughout the year in endemic areas, which implies that AHSV transmission could occur throughout winter *(167)*. Once infected after feeding on a viraemic animal, the *Culicoides* female will remain persistently infected and able to transmit virus with each subsequent blood meal. A females' lifespan is on average <20 days, but can under the right conditions be as long as 90 days *(115)*. This means that it is possible that an AHSV infected adult female could survive a short winter, providing a mechanism for viral overwintering. A progressive change in climatic zones, and the potential spread of the vector of AHS, together with the increase of movement of horses around the world, has increased the risk of international spread of AHS *(50, 136, 158)*. Other research indicates that the geographic range of the *Culicoides* midge has not shifted significantly *(41)*, however this does not alter the fact that appropriate *Culicoides* spp. occur in areas currently free of AHS, and therefore are potential vectors for transmission should AHSV be introduced.

AHS was first identified in South Africa in 1719, during a major AHS outbreak in the Cape Colony after horses were introduced to the region. During this outbreak over 1 700 animals died. At least 10 other major outbreaks of AHS have been recorded in southern Africa since the original recognition of the disease, the largest being in 1854–1855 with more than 70 000 horse fatalities *(17, 74)*.

AHS is endemic in tropical and subtropical areas of Africa, south of the Sahara, from Senegal in the west to Ethiopia and Somalia in the east and extending as far south as southern Africa *(114)*. Epidemics of AHS have occurred in North African countries on a number of occasions following spread of the disease up the west coast of Africa or down the Nile valley. AHS epidemics have also occurred in the Middle and Near East (1944 and 1959 to 1963) and southern Europe (1966 and 1987 to 1990). The 1987 AHS epidemic in southern Europe was associated with the introduction of infected zebra from Namibia to a safari park in Spain *(60, 184)*.

In South Africa, outbreaks of AHS occur every summer throughout the country with the exception of the region of the southern Cape. Historically, the area around the Cape of Good Hope has been free of AHS with sporadic outbreaks caused by movement of AHS infected horses into the area. This led to the establishment of an AHS controlled area in accordance with European Union legislation in 1997 in order to facilitate export horses from South Africa. The AHS controlled area comprises of a free, surveillance and protection zone (Figure 1-1) and was implemented by South African authorities in the same year *(16, 157)*. Since the



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implementation of this regionalisation outbreaks within the surveillance zone have occurred in 1999, 2004, 2011, 2013, 2014 and now more recently in 2016 as reported to the OIE *(179)*. The increase in frequency of the outbreaks in the AHS controlled area has had a negative impact on the equine export industry of the country, as well as raising serious concerns relating to the increase in spread of disease as well as the increase in frequency of outbreaks in historically free areas.

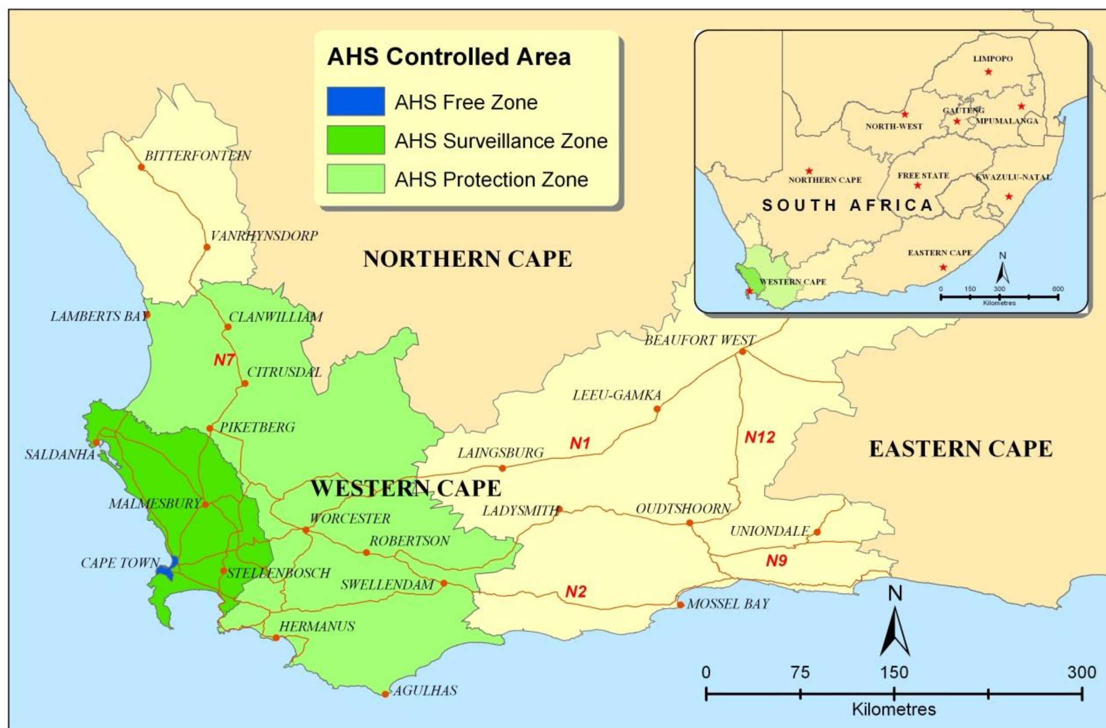


**Figure 1-1: A map of the zones within the African horse sickness (AHS) Controlled Area of South Africa.**

## 1.2 Pathogenesis

After infection of a susceptible equid through the bite of an infected vector, initial replication of AHSV occurs in the regional (draining) lymph nodes. Thereafter, a secondary viraemia occurs where the virus disseminates to the endothelial cells of the target organs, namely the lungs, spleen and other lymphoid organs *(62, 114)*. Viral replication then occurs in these organs. Endothelial cell damage appears to be central to the pathogenesis of AHS. The damage is thought to be due to cellular injury resulting in ultra-structural changes in, and separation of, the endothelial cells *(52, 92)*. This leads to effusions into body cavities, oedema and serosal and visceral haemorrhage *(62)*. However, one study showed that there was negligible morphologic damage to the pulmonary endothelial cells *(120)*. This has led to



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speculation that inflammatory mediators play a role in the pathogenesis of AHS, perhaps leading to endothelial activation and retraction that result in enhanced vascular permeability.

The virus is closely associated with erythrocytes in the blood *(163)*. High concentrations of virus are found in the lymphoid tissue, and this might explain the lymphopaenia seen in experimental cases of AHS *(44, 156)*. On a molecular level the likely series of events for cell entry are as follows:

- proteolytic cleavage of VP2 in serum or midge saliva.
- interaction with host cell receptor via VP2.
- VP5 activated leading to exposure of fusion peptide.
- release of core into cytoplasm *(49)*.

Virulence of AHSV is thought to be related to the affinity of the virus for certain tissues and attenuation is simply the selection of viruses that do not have selective affinity for vital organs (e.g. lung) *(44)*.

## 1.3 Clinical signs

The clinical symptoms of AHS have been well documented *(163)*. The disease is characterised by pyrexia, oedema of the lungs, pleura and subcutaneous tissues, as well as petechiae and haemorrhages *(88)*. The incubation period in experimental cases on average is five to seven days *(30)*. In a recent study where a horse was inoculated with a virulent field strain of AHSV, the incubation period was seven days before dsRNA was detected by RT-qPCR *(137)*.

The viraemic period corresponds with the onset of fever *(92)*, and can persist for between two to fourteen days *(77)*. This has been confirmed by viral isolation *(44, 147)*. The exact period that the viraemia can extend beyond this time period is unknown, as viral isolation is not always sensitive enough to detect lower titre viraemia *(147)*. It has been shown that AHSV dsRNA, detected by RT-qPCR, peaked at approximately 15 days post-infection and persisted for up to 97 days after initial exposure to the virus *(137)*. Further studies are necessary to determine the period during which horses are infectious to midges and the relationship between this and the persistence of viral nucleic acid detected using RT-qPCR. Given the erythrocyte-associated viraemia of AHSV in horses and the approximately 145 day lifespan of the red cell in circulation in the horse *(93)*, the persistence of RT-qPCR positive results could simply be related to the red cell life span.



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There are four clinical forms of AHS. The peracute, or pulmonary form, syn. "dunkop", is characterised by an incubation period of three to five days. The clinical signs are those of fever (39.5 – 40.5°C), followed by congestion of the mucous membranes. The animal becomes depressed, with dyspnoea, sweating and coughing a few hours prior to death. Some cases may also discharge copious amounts of frothy, serofibrinous fluid from the nostrils, which may occur only after death. Mortality is around 95%. Fully susceptible horses, such as foals that have lost their colostral immunity or unexposed horses, usually suffer from this form *(30, 62, 80, 114, 116)*. The subacute or cardiac form, syn. "dikkop", has an incubation period of seven to fourteen days. Fever (38.5 – 39.5°C) and mucous membrane congestion are the initial signs, and later, subcutaneous and intermuscular oedema, particularly of the head and neck, occurs. Supraorbital swelling due to oedema is characteristic of the cardiac form. Petechiae may occur on the ventral surface of the tongue and conjunctivae and usually occur just prior to death. The presence of petechiae indicates a poor prognosis. Mortality is around 50%. The acute or "mixed" form is suspected to be the most common form, and presents with clinical signs of both the pulmonary and cardiac forms. The "Horse sickness fever" form of AHS is mild with an incubation period of five to nine days. It is suspected to occur in partially immune animals, and manifests as a low-grade fever with mild depression. This form may be more prevalent as these mild symptoms are often not observed. Owners may not report these findings to a veterinarian, as the horse has usually recovered after being slightly "off" for a day or two, and they therefore do not seek veterinary attention. This is the most common form seen in zebras and donkeys, which are resistant to the development of clinical signs *(30, 62, 80, 88, 114, 116)*.

## 1.4 Macroscopic pathology

On post mortem the outstanding feature of severe AHS is that of oedema. In the "dunkop" form there is marked pulmonary oedema, severe hydrothorax and petechiae. In the "dikkop" form the characteristic finding is the presence of a slightly yellowish, gelatinous oedema of the intermuscular connective tissues and subcutaneous tissues of the head and neck that is often particularly severe around the ligamentum nuchae. A severe hydropericardium is often present. In the "mixed" form a mixture of the "dunkop" and "dikkop" changes are found *(62, 88)*.

## 1.5 Clinical pathology

Whilst the clinical signs and presentation of AHS have been well documented by Sir Arnold Theiler in 1921, very little has been published regarding the clinical pathologic findings of the



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disease. Haematological abnormalities seen under experimental conditions include a leukopaenia (specifically a lymphopaenia and a neutropaenia), a thrombocytopaenia and an elevated haematocrit (Ht), red cell count (RCC) and haemoglobin (Hgb) concentration *(156)*. In the study carried out by Skowronek et al *(156)* margination of leukocytes within pulmonary vessels and increased leukocyte numbers within capillaries were observed histologically and probably contributed to the leukopaenic state. Other contributors could be stress-induced corticosteroid release with subsequent lymphocyte sequestration and viral interference with leukocyte production *(93)*. The polycythaemia seen as an increase in Ht, RCC and Hgb, was most likely due to compromised endothelial cell barrier function and a subsequent shift in fluid from intravascular to extravascular compartments. Febrile dehydration may be a possible explanation for the polycythaemia. Haemostatic abnormalities included increased fibrin degradation products, prolonged prothrombin, activated partial thromboplastin and thrombin clotting times *(156)*. A decrease in thromobocyte count has been reported experimentally and in the field *(156, 176)*. In a field study done by Weyer et al *(176)*, it was shown that the thrombocyte count is related to the RT-qPCR quantitative cycle (Cq) value, with a lower Cq value (higher amount of RNA) corresponding to a lower thrombocyte count (greater thrombocytopaenia). It has been suggested that the thrombocyte count, together with the Cq value could be used as a prognostic indicator for AHS.

## 1.6 Control

### *Vaccination and immunity*

The first AHS live attenuated vaccine (LAV) was developed in the 1930's using serial passage in mouse brain *(6)* and has since been replaced by tissue culture attenuated vaccine viruses *(77, 172)*. Virus strains included in the vaccine are attenuated through multiple suckling mouse brains or cell culture passages followed by plaque purification to select mutants that are non-pathogenic but immunogenic *(45, 172)*. Although the vaccine is relatively cheap, and on the whole provides adequate immunity, it has a number of limitations. It has the potential for variable attenuation and a possible lack of immunogenicity. There is a variable immune response in individual animals to each strain, which may be due to over attenuation of certain strains, or interference between strains in the vaccine *(30, 42)*. There is also a possibility of reversion to virulence of the AHSV-LAV strains *(126)* and reassortment between strains within the vaccine and with wild/ field strains. The vaccine can interfere with laboratory diagnostic tests, as the vaccine virus cannot easily be distinguished from natural infection in the horse *(63, 77, 80, 100)*.



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In southern Africa, a polyvalent cell culture attenuated vaccine is currently available commercially from Onderstepoort Biological Products (OBP). Horses that have received three or more annual courses of vaccination are considered to be sufficiently protected *(42, 62, 172)*. Despite the rigorous use of vaccination to control AHS, cases of AHS occur annually in certain high-risk areas (including Onderstepoort). There are also anecdotal reports that vaccinated horses can become infected with AHSV without developing clinical signs of infection *(83)*. Such animals may provide a source of virus for vector midges and as such may well play a role in the spread of the disease if such animals are relocated during the infectious period.

The current vaccination schedule recommended by the manufacturers of the OBP polyvalent live attenuated vaccine is as follows *(65)*:

| | |
|---|---|
| **Initial vaccination:** | Weanlings at approximately 6 months of age |
| **Secondary vaccination:** | Yearlings at approximately 12 months of age |
| **Annual booster:** | All horses in high-risk areas should be vaccinated in late winter or spring (Sept-Nov). |

The OBP polyvalent vaccine is made up of two components: OBP comb1 and OBP comb2, with each component given at least three weeks apart. OBP comb1 is a trivalent vaccine and contains types 1, 3 and 4. OBP comb2 is a quadrivalent vaccine, and contains types 2, 6, 7 and 8. Type 8 provides cross-protection against type 5 and type 6 cross-protection against type 9 *(172)*. Although these vaccines should theoretically provide a lifelong immunity and a single dose should be sufficient, annual vaccination is advised to try to produce true polyvalent immunity in the face of variations in response to the vaccine. It has also been shown that wild-type virus infection appears to induce a more broadly cross-reactive immunity than administration of attenuated vaccine virus *(20)*.

One of the concerns related to live virus vaccines is the possibility of reversion to virulence by mutation and reassortment. Furthermore, it is believed that polyvalent virus vaccines present a greater potential for reassortment due to the co-administration of different strains which are then co-multiplying in the host and creating a pool of genomic segments with the potential to reassort with one another.

Inactivated vaccines have also been used in outbreaks of AHS in non-enzootic regions; however these vaccines are no longer available commercially. Inactivated vaccines have the



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advantage of being very safe, but require repeated immunisations because of loss of immunogenicity *(37, 80)*. Virus like particles (VLPs) produced from recombinant baculoviruses *(142)*, a DNA vaccine *(141)*, and recombinant poxvirus vectored vaccines *(3, 63)* are among other vaccines that have been developed to combat AHSV infection in horses. VLPs are safe and have been shown to provide adequate protection experimentally, but have not been used in the field due to commercial production difficulties and cost as well as problems with long-term stability *(63, 143, 151)*.

A recombinant canarypox-vectored vaccine against AHS type 4 has been developed recently *(63)*. It appears to be safe and produce solid protective immunity against this type *(39)*, and has none of the disadvantages described for live attenuated vaccines. This vaccine was constructed using synthetic genes encoding the VP2 and VP5 proteins of AHSV-4 inserted into a recombinant canarypox virus vector.

Most AHSV vaccines developed recently have been shown to effectively induce neutralising antibody responses in horses, which has historically been described as a good marker for protection *(39)*. However there have been studies that have reported significant neutralising antibody titres following vaccination but were found to be unprotected against virulent virus *(108)* and conversely in the same study a horse that had undetectable neutralising antibodies that survived subsequent challenge. Guthrie et al *(63)* reported similar findings where a horse that did not seroconvert after vaccination was protected against challenge. Cellular mediated immunity has been suggested to play a role in protection in the form of virus-specific CD8+ T-cells, otherwise called cytotoxic T-cells *(39, 108, 134)*.

Foals born to immune mares acquire passive immunity by ingestion of colostrum after birth. This immunity declines progressively as a result of catabolism of immunoglobulins to undetectable levels at four to six months of age *(5, 30, 127)*. Foals are routinely vaccinated at around 6 months of age and not before, as it has been shown that vaccination in the presence of maternal antibody can lead to weakened antibody responses, although it appears that it does not adversely affect the pre-existing maternal antibody *(20)*. Passively acquired maternal antibody to the different AHSV types varies considerably among foals born to immunised mares *(33)*.

***Management***

In southern Africa, vaccination alone is not fully effective in the control of AHS. Managemental precautions need to be implemented as well. Stabling at night, especially from the time before sunset to after sunrise, is helpful, as this is considered the peak activity



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time for midges associated with AHSV transmission. The *Culicoides* spp. present in southern Africa do not readily enter buildings *(14)*. Topical insect repellents and insecticides applied to the horse are also effective. Control needs to be looked at as a holistic approach, rather than relying on one or the other control measures in isolation.

## 1.7 AHSV Structure

The AHSV virion is an unenveloped double-layered particle of about 70nm in diameter demonstrating icosahedral symmetry *(128)*. The genome consists of ten double-stranded RNA segments *(25, 122)* housed within a capsid *(144)*. These segments encode seven structural proteins (VP1-7; Figure 1-2) and five non-structural proteins (NS1, 2, 3/3a and NS4). The outer capsid consists of 2 of these proteins (VP2 and 5). The inner core comprises two major proteins, VP3 and VP7, both of which are highly conserved *(114, 137)* and 3 minor proteins (VP1, VP4 and VP6) *(144)*. The designation of the various proteins and gene segments of different orbiviruses based solely on their size is confusing, and a nomenclature system based on sequence homology and functional characteristics is therefore preferable, as described by Dilcher and Weidemann *(35)*.

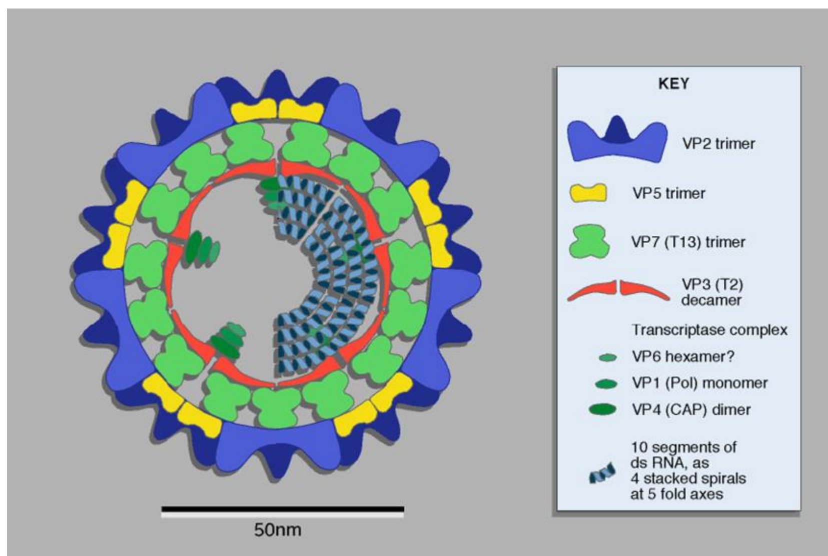


**Figure 1-2: A diagrammatic representation of the structure of the African horse sickness viral particle, showing the positioning of the seven structural viral proteins (VP1-7) and the segmented RNA genome (after Wilson et al. 2009).**

### *Minor inner core proteins*

VP1 (Pol), the RNA dependant RNA polymerase, VP4 (CaP), the capping enzyme and VP6 (Hel), ssRNA and dsRNA binding helicase, may aid in the mRNA transcription process *(35,*



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*143, 159)*. The VP1 (Pol), VP4 (CaP) and VP6 (Hel) are all present in low amounts in each particle and are associated with viral RNA synthesis and capping.

***Major inner core proteins***

VP3 (T2) which is the innermost component of the capsid also has group specific antigenic properties *(144)* and is highly conserved between AHSV types.

VP7 (T13) is the major component of the inner core. VP7 contains group-specific antigenic determinants and is highly conserved within the orbiviruses *(144)* and so this protein is suitable for serological and molecular diagnostics at a serogroup (virus species) level.

***Outer Capsid proteins (VP2 and VP5)***

VP2 protein is the principal outer capsid protein, and is type specific as it includes the major neutralising epitopes of AHSV *(132, 144, 173)*. VP2 also exhibits considerable variability between AHSV types *(10)*. VP2 is also thought to mediate initial viral cell attachment *(102)*. A truncated VP2 has been described in AHSV type 7, which is caused by an in-frame deletion in the genome segment L2 that encodes VP2 (225 amino acids, residues 279 to 503, deleted) *(102)*. Amino acid residues 340 to 360 of VP2 (which fall within this deleted section) are implicated in determining tissue tropism and virulence *(132)*. VP2 is sensitive to serum proteases which can result in cleavage of VP2 in the outer capsid leading to modification of biological properties and solubility of the particle *(159)*. The cleavage of VP2 by proteases has been shown to increase infectivity of the virus to *Culicoides (103)*. It is also likely that proteases in the insect's saliva could lead to VP2 cleavage *(34)*.

VP5 contains coiled-coil motifs which are typical of membrane fusion proteins responsible for membrane penetration for cell entry *(102)*. Although located on the outer capsid, this protein is seemingly less exposed at the surface of the virion than the VP2 protein, and does not appear to be involved in AHSV antibody-mediated neutralisation *(144)*.

***Nonstructural Proteins***

Following viral adsorption and endocytosis, the outer capsid proteins are removed and the core particle is released into the cytoplasm of the infected cell *(166)*. Viral replication then occurs within the cytoplasm of the cell. AHSV infection results in the formation of viral inclusion bodies and the assembly of thin tubular structures in the cytoplasm *(166)*. The tubular structures are comprised of multimers of NS1 (TuP), which is highly conserved



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among types *(144)*. The matrix of the viral inclusion bodies is made up of NS2 (ViP), which provides the scaffold for viral replication and assembly, recruiting mRNA during virus assembly *(144)*. NS3/3a is involved in virus release from infected cells *(144)*. The recently discovered NS4 is thought to be involved in viral replication. This non-structural protein appears to modulate the host's innate immune response to infection and counteract the cellular antiviral response in interferon-treated cells as seen in BTV *(188)*. NS4 is expressed in AHSV-infected mammalian cells.

## 1.8 Viral reassortments and reversion to virulence

The AHSV genome consists of 10 segments of double-stranded RNA which encode for the structural and non-structural proteins, surrounded by the viral core. In intact virus particles this core is enclosed with an outer capsid (VP5 and VP2). The outer capsid proteins are involved in cell attachment and penetration during infection in mammalian cells. Following adsorption of the virus is taken up by endolysosomes and penetrates the cell membrane in a process known as receptor-mediated endocytosis. VP2 is removed from the virus while in these acidic endosomes, following which the partially uncoated virus particle is released into the cytoplasm where VP5 may also be removed. Once entry into the target cell is achieved and the core particle is released into the cytoplasm, the transcription process is activated. The processes of AHSV transcription, replication and morphogenesis are likely similar to that of BTV *(53, 178)*.

The segmentation of orbivirus genomes allows for the exchange of genome segments between orbiviruses of the same serogroup but belonging to different types or originating from different geographical regions, when cells are infected simultaneously by at least two viruses *(153, 161, 170, 184)*. Reassortment can lead to altered pathogenicity of individual virus strains, for example reassortment could lead to increased pathogenicity (virulence) of a previously apathogenic strain. This would be important especially in the development and use of live attenuated vaccines, such as the one commercially available in South Africa. Reassortments could potentially occur between vaccine strains or vaccine and wild strains if more than one strain is present in the horse cell at the same time *(153)*. Reassortments could also lead to a difference in transmissibility, allowing a virus to increase the capability of transmission and therefore increasing the potential for an outbreak *(170)*. Data suggest that *in vivo* reassortment occurs more frequently in the insect than in the vertebrate host and are scarcer to document for AHSV than for BTV in vertebrate hosts *(178)*.



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A major concern with all live, attenuated vaccine viruses is the potential for reversion to a previously pathogenic parent strain. When considering differences in virulence and transmission, and therefore reversion to virulence or attenuation, it is likely that the more variable genome segments are involved; those encoding VP2, VP5 (outer capsid proteins) and NS3 *(29, 84)*. The virulence of a virus is most likely related to the tissue tropism of the virus. Considering that the outer capsid proteins are involved in cell entry and release into the cytoplasm *(71, 72)* it makes sense that these proteins would then be related to virulence *(132)*. NS3 has been shown to be a key protein in AHSV release from the infected cells and membrane permeability alteration *(111)*. This then could determine the speed of spread of the virus within and between organs *(84)*.

## 1.9 Diagnostic Techniques

Clinical signs, history and macroscopical lesions are usually sufficient to suggest a diagnosis of AHS; however these clinical signs and lesions are not specific for AHS. The clinical symptoms of AHS, especially the “horse sickness fever” form are very similar, if not identical to that of equine encephalosis virus (EEV). Some horses with EEV also exhibit the subcutaneous and intermuscular oedema, as well as the supraorbital swelling. The recumbancy and peracute death as well as the fever seen in cases of “dunkop” can be confused with the symptoms of West Nile virus (WNV). Terminal cases of WNV, however, usually exhibit typical neurological signs, which are not present in AHS. In some cases, pyrexia is the only symptom, and there are many differential diagnoses for this symptom. For these reasons laboratory confirmation of infection with AHSV is essential. A diagnosis can be obtained by identification of infectious virus, viral antigens or specific antibodies *(183)*. A number of such tests have been developed for confirmation of infection with AHSV.

### *Agent Identification*

#### *Viral Isolation and Identification*

Traditionally virus isolation with serotyping is used to provide a definitive diagnosis of AHS. Blood should be collected in heparin during the febrile stage, or lung, spleen and lymph node specimens collected at necropsy and kept at 4°C. High viral levels are usually found in the above organs of fatal cases *(30)*. Viral isolation may be obtained by inoculating the test sample onto a variety of cell cultures (e.g. BHK-21, Vero) or by intracerebral inoculation of suckling mice *(183)*. In cell culture, the cytopathic effect (CPE) of the virus is measured by refractivity and detachment of cells, and can appear two to eight days post-inoculation. Once



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it is clear that a virus has been isolated, it must be confirmed as AHSV, as the morphology and cytopathic effects of EEV are almost indistinguishable from AHSV *(31)*. To a lesser extent the same can be said for WNV.

Processed blood samples are inoculated onto a confluent monolayer of BHK-21 cells and incubated at 37°C. Cultures are observed daily for any CPE. When 100% of the cell monolayer show CPE the cells and supernatant are harvested and identification is confirmed as AHSV using methods described below Cultures showing no CPE after three passages are classified as negative for AHSV *(138)*.

*Indirect sandwich ELISA*

Specific antibody (rabbit hyperimmune serum) is adsorbed to the ELISA plate. A test sample is added to the plate, allowing antibody–antigen complex formation. Antibody produced in a different host (guinea pig immune antiserum) is then added to the plate, which binds to the original antibody-antigen complexes allowing antibody-antigen-antibody complex formation. An enzyme-labelled species-specific antiserum (rabbit anti-guinea pig antibody, conjugated to horseradish peroxidase) directed against the antibody of the second species is added. A chromogen (ortho-phenylene diamine) and substrate (0.05% $H_20_2$) are added, which leads to a colour change in the presence of antibody-antigen-antibody-enzyme conjugate complexes. The reaction is stopped by adding $H_2SO_4$ after 5 – 10 minutes, and the absorbance value is measured spectrophotometrically. The plate is washed removing any unbound reagents and incubated at prescribed temperatures after each step. The cut-off value is the absorbance value obtained from the negative control. Any samples with an absorbance value lower than the cut-off are regarded as negative. Test samples with values greater than the cut-off are regarded as positive *(69)*.

*Virus Neutralisation*

Virus serotyping is done using a plaque inhibition neutralisation test using type specific antisera. It can be used to identify unknown viruses with the use of a positive, known serum. It can be used to find the type of a virus that consists of several distinct types, as is the case with AHSV *(183)*. Electrical insulating fish-spine beads filled with type-specific antiserum (produced in sheep) are used to indicate virus-antibody neutralisation on Vero cell monolayers inoculated with the test sample *(130)*. The fish-spine beads are placed on the surface of the inoculated Vero monolayer. A neutralisation zone around the bead is indicated by an absence of plaque formation as shown in the example in Figure 1-3. *(81)*.



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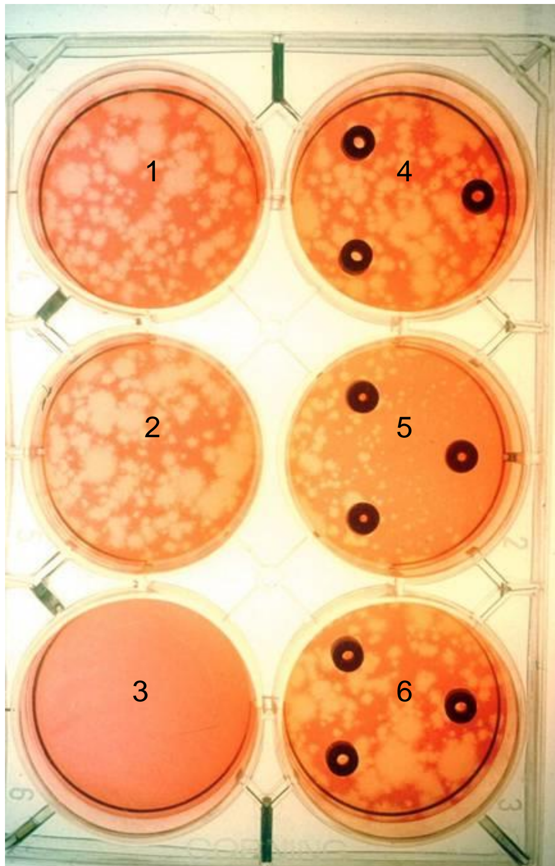


**Figure 1-3: The plaque inhibition neutralization test: wells numbered 1-3 are the controls. Wells numbered 1 & 2 show viral plaque growth (multifocal pale areas) and well 3 is the negative control with no inoculated virus on a monolayer of Vero cells. Wells numbered 4-6 show monolayers with fish-spine beads (three beads per well) with antiserum to a different type in each bead. Well 5 shows a zone of inhibition around the right most bead where antibody neutralisation of AHSV has occurred.**

The plaque inhibition neutralisation test: wells numbered 1-3 are the controls. Wells numbered 1 & 2 show viral plaque growth (multifocal pale areas) and well 3 is the negative control with no inoculated virus on a monolayer of Vero cells. Wells numbered 4-6 show monolayers with fish-spine beads (three beads per well) with antiserum to a different type in each bead. Well 5 shows a zone of inhibition around the right most bead where antibody neutralisation of AHSV has occurred.

*Group specific PCR*

One of the disadvantages of viral isolation and viral neutralisation tests for virus typing is that it usually takes a minimum of two weeks to obtain a result *(69, 146)* and this time constraint can be costly in the face of an outbreak situation, where a rapid, sensitive, specific and reliable test is needed. A number of polymerase chain reaction (PCR) assays have been developed for the detection of AHSV targeting the VP3 *(9)*, VP7 *(186, 187)*, NS1 *(118)*, NS2 *(158)* or NS3 *(185)* genes. These tests have the potential to be rapid, sensitive and versatile, and can supplement the older conventional methods. They can be used on specimens that



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do not contain live virus *(1, 30)*, that contain attenuated strains or strains of low virulence *(147)* and can be used for earlier detection of viraemia than viral isolation *(158)*. However none of these assays have been optimised for the detection of AHSV in blood from naturally infected horses *(137)*. The majority of AHSV assays are based on visualisation of the PCR product on agarose gel. Advantages of a real-time PCR over a gel-based PCR are that the real-time has a greater analytical sensitivity and specificity, is quicker to perform, and has a smaller potential for contamination *(75)*. A RT-qPCR has been developed recently in the Equine Research Centre (ERC) laboratory, University of Pretoria. This assay has been applied to samples collected from clinical field cases of AHS and has been applied to serial samples collected from animals experimentally infected with AHSV. This assay is unique in that it is designed from sequences of current circulating field virus strains *(137)*.

This RT-qPCR assay was used in this prospective study to quantify the AHSV viraemia. In brief, nucleic acid was extracted from 100 µl of blood and then subjected to RT-qPCR using specific primers and probes for the genes coding for the VP7 protein of AHSV. RNA purification was done using MagMAX™ Viral RNA Isolation kits (Ambion) and a Kingfisher 96 Magnetic Particle Processor (Thermo Scientific) according to a protocol supplied by the manufacturer of the kit. The sample underwent a lysis step and two washing steps and was finally eluted in 90 µl elution buffer.  The eluate together with primers and probes (targeting AHSV VP7 genes and Xeno RNA) was heat-denatured. VetMax™-Plus Multiplex One-Step RT-PCR kit (Lifetech) reagents were then added and a one-step RT-qPCR performed on a StepOnePlus™ Real-Time PCR System (Lifetech) using a protocol as recommended by the manufacturer. This assay is a rapid, analytically sensitive and specific assay for detection of AHSV RNA *(137)*.

The Kingfisher 96 Magnetic Particle Processor (Thermo Scientific) uses magnetic rods to collect the magnetic beads included in the MagMAX™ Viral RNA Isolation kit (Ambion) and enables high throughput RNA isolation. Traditional methods, such as phenol: chloroform extraction, are time consuming and difficult to automate and perform. High throughput techniques use glass fibre filters or magnetic beads. The magnetic beads provide higher purity RNA with more consistent recovery *(46)*. A guanidinium thiocyanate-based solution is used to solubilise cell membranes and inactivate nucleases, releasing the viral RNA. The magnetic beads with nucleic acid binding surfaces are then added, which bind the nucleic acid. The beads and RNA are captured on the magnets and washed to remove cellular debris and proteins. They are then washed again to remove residual binding solution. The RNA is then eluted in a low salt buffer solution, which releases the RNA from the magnetic beads *(183)*.



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Xeno RNA control (Lifetech) is included as an internal control for the RT-qPCR, which undergoes independent amplification, increasing reliability of the test. A positive result for Xeno RNA implies that extraction of the RNA has been completed successfully and that no PCR inhibitors are present *(75)*. The inclusion of this internal control provides a method to identify false negative results due to unsuccessful extraction or the presence of PCR inhibitors. PCR inhibitors include substances such as the porphyrin ring of haem found in haemoglobin of red blood cells, heparin and chelating agents such as EDTA *(12)*. These inhibitors are important to note, as the samples submitted for the RT-qPCR are generally either heparin or EDTA blood samples.

The diagnostic accuracy of this assay has been determined *(61)* and this RT-qPCR with viral isolation is used for AHSV detection in the laboratories at the Faculty of Veterinary Science of the University of Pretoria.

*Type Specific RT-qPCR*

Determination of AHSV type can be important to both epidemiological studies and vaccination strategies. For example, outbreaks of AHS among horses in the AHS-controlled area in the Western Cape region of South Africa have highlighted the need for rapid and specific determination of the type of the incurring virus. AHSV type-specific real-time RT-PCR (ST RT-qPCR) assays offer the potential for more rapid determination of the virus type involved in such outbreaks. AHSV TS assays that target the portion of the L2 gene encoding the major neutralisation determinants of AHSV *(27, 86, 133, 173)* are well suited for accurate, rapid type determination. Direct TS RT-PCR assays have been developed *(146)*; however these assays are cumbersome and time consuming as they require the use of gels for assay confirmation. Direct TS RT-PCR assays also increase the risk of contamination and false positive results. Koekemoer (90) developed an AHSV TS RT-qPCR assay that utilizes a sensor probe and an anchor probe on two different channels, followed by melt curve analysis to differentiate the various AHSV types. Although these assays accurately confirmed the type of historic prototype South African strains of AHSV, they showed considerable variation in determination of the AHSV type of field strains. More recently, TS RT-qPCR assays for the detection and typing of AHSV have been developed and described *(10)*. All assays described above require 9 separate PCR assays in order to type the virus. This is not practical for routine application and screening in endemic areas, or in outbreak situations where prompt and accurate disease control measures must be applied. There is therefore the need for accurate, quick and practical methods for outbreak etiological confirmation.



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*Whole Genome sequencing*

Recently great advancements in cloning, amplification and sequencing of genome segments of dsRNA has led to techniques being developed which allow full-length sequences of viral dsRNA genomes to be obtained *(7, 51, 96, 131, 132)*. RNA can be extracted from tissues and converted into complimentary DNA (cDNA) by the process of reverse transcription. cDNA can then be subjected to amplification and then sequenced in the same way as genomic DNA. This technology can be used to investigate and reveal viral segment reassortments. The information gained from these sequences can be used to investigate viral attributes such as viral tropism and virulence. It can also be used to study viral evolution of outbreaks in the field, assessing attenuation and adaptation to cell culture *(132)*.

Basic whole genome sequencing methods include that of the chain-termination method (Sanger sequencing). Sanger sequencing has been the method of choice for whole genome sequencing since the late 1970s until recently (mid-2000s) owing to its relative ease and reliability. This method has been developed by Applied Biosystems based on the selective incorporation of chain-terminating dideoxynucleotides triphosphates (ddNTPs) by DNA polymerase during *in vitro* DNA replication *(150)*. The DNA sequence is read through fluorescent emission during ddNTP binding.

More recently next generation sequencing (NGS) techniques have been developed. At present there are two commonly used commercially available NGS techniques. Roche Diagnostics have developed the 454 pyrosequencing technique *(132, 152)*. In short DNA is amplified inside a water droplet in an oil solution (Emulsion PCR) with each droplet containing a single DNA fragment. This technique uses enzyme cascades and luminescent detection to measure the release of inorganic phosphate upon incorporation of the next complimentary nucleotide *(85, 132)*. Illumina sequencing is based on DNA clusters or colonies which are created by clonal amplification of DNA on a solid surface (slide). To determine the sequence of the DNA fragments of these clusters four reversible terminator nucleotides with fluorescent tags area added. With this technique the sequence is extended one base at a time *(104)*. The sensitivity of the method used depends greatly on the level of viraemia in the original sample and the amount of dsRNA that can be extracted from the sample *(132)*.

*Nucleotide and Protein Sequence Analysis*

The first step in nucleotide and protein sequence analysis involves multiple sequence alignment; the process of aligning a number of biologically relevant length, allowing one to



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compare the identity between different sequences. Historically this was done manually but is time consuming and difficult. Computer algorithms have now been developed for this purpose, among them MAFFT and Clustal *(87, 164)*. Sequences are loaded into the computer programme from a database (eg *GenBank*) which performs the alignment.

Once the sequences are aligned phylogenetic analysis can be performed by creating phylogenetic trees which depict evolutionary relationships between a set of sequences. Trees are about groupings or clades. There are two general methods for calculating trees; distance-matrix methods and discrete data methods. Distance methods are calculated by calculating the percentage sequence difference for all pairwise combinations and then the percentages (distances) are assembled into a tree (e.g. neighbour-joining). Discrete methods examine each column of the aligned sequences and then creates a tree to accommodate this information (parsimony, maximum likelihood, Bayesian methods) *(11, 59)*. An example of such a programme is RAMI, which aims to identify groups or clades in phylogenetic trees based on patristic distances *(129)*. Patristic distances are calculated by assessing the amount of genetic changes between sequences. To test the accuracy of your phylogenetic tree bootstrapping is used, which tests the level of confidence for each clade in an observed tree *(38)*. This is basically done by resampling or analysing subsets of your data and building trees from this subset and calculating the frequency with which the various clades or branches of your tree are reproduced *(11, 89)*. Bootstrap values of 70% of higher generally indicate reliable groupings.

However, as mentioned previously the AHSV has a segmented genome structure. This means that should there be co-infection of a cell with multiple AHSV strains, there can potentially be a mixture of genetic material between individual viruses of different lineages (segment reassortment). This phenomenon is termed horizontal evolution (as opposed to vertical evolution) and can create incompatibilities that mischaracterise trees. This genomic structure is a feature that is shared with other viruses such as the influenza viruses, rotaviruses and of course other orbiviruses such as bluetongue virus. The benefit however, is that the genome sequence can be analysed per segment, and a model for reassortment hypothesised by analysing the relationships of each segment with other known segment sequences *(161, 170)*.

The development of automated computer programmes in order to detect and analyse recombination and reassortment events has led to more accurate analysis and the ability to analyse large sets of data. Recombination detection programmes, given a set of aligned nucleotide sequences, can identify and characterise individual events by sequentially testing



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every possible combination of three sequences for evidence of recombination or reassortment *(161, 170)*. RDP4 is an example of such a programme *(106)*.

Another possibility in viral genomics would be the reversion of an attenuated strain, such as a AHSV-LAV strain, to the original parent, pathogenic strain. This occurrence can be evaluated and confirmed by looking for single nucleotide variants (SNVs). This is done by identifying genome positions with nucleotide or more likely amino acid variations relative to a reference *(124)*. Again there are many programmes available today that can do large scale analysis for SNVs *(89, 124)*.

***Antibody identification***

*Group-Specific Serology*

Group specific antibodies can be detected using complement fixation tests (CFT), agar gel immunodiffusion (AGID) tests, indirect immunofluorescent antibody (IFA) tests and ELISA tests.

The CFT uses an immunological indicator system (a haemolytic system) to detect immune complex formation, and therefore group specific antibodies. The haemolytic system consists of red blood cells (RBC), normally harvested from sheep, and anti-red blood cell hyperimmune serum (rabbit serum). The test uses the concept that RBC's in the presence of hyperimmune serum will form immune complexes, which will trigger the complement cascade leading to lysis of the RBC's, which is a visible reaction.

The first step of the test is to incubate a known antigen (sucrose/ acetone extract of AHSV-infected mouse brain) with test, known positive and known negative samples. Immune complexes form. Complement in the form of guinea pig serum is added. If immune complexes are present, the complement will be consumed. The haemolytic system is then added. If no complement is left, i.e. the immune complexes formed in step one, then no haemolysis will occur. If no immune complexes have formed, then the haemolytic system will trigger the complement cascade, and haemolysis will occur *(110, 183)*.

The CFT detects primarily IgM antibodies, and is therefore ideal for detection of the primary antibody response *(31)*. However some sera may have an anti-complementary effect and the use of this test is becoming less popular.



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AGID tests utilise precipitation reactions in a semisolid medium. Through diffusion, optimal concentrations of antigen and antibody are brought together, and when the concentration of the complexes exceed that of the gel's capacity, the complexes precipitate out and form visible bands *(70, 78)*. The antigen used in this test is crude and non-specific lines are often obtained which can interfere with accurate diagnostics *(78)*.

The indirect IFA utilises a standardised antigen and fluorescent antiglobulin to determine the presence of a specific antibody. The fluorescent antiglobulin binds to antigen-antibody complexes and fluoresces. Non-specific fluorescence, especially in vaccinated horses, occurs often, which may make interpretation of results difficult and lead to false positive results *(78)*. For this reason, the test is better used as a screening test.

An indirect ELISA using either soluble AHSV antigen or a recombinant VP7 protein can be used. These assays detect IgG antibodies to either the soluble antigen or the VP7 protein. The indirect ELISA, using recombinant AHSV VP7 antigen which is conserved among types *(62, 114)*, is more sensitive in detecting early immunological responses when compared to other assays. It can be used to detect declining levels of maternal antibodies and has a good specificity *(105)*. Other advantages of the use of VP7 are that it is stable and not infective *(183)*. It is considered a good screening test for unvaccinated animals and is listed as a prescribed test for international trade by the OIE Terrestrial Manual *(183)*.

The indirect ELISA performed at the Agricultural Research Council - Onderstepoort Veterinary Institute, is described by Maree and Paweksa, 2005. To perform the test antigen (AHSV recombinant VP7 protein) is adsorbed onto the ELISA plate. The test and control sera are added and antigen-antibody complexes form. An enzyme-conjugate (recombinant protein G conjugated with horseradish peroxidase) is added which binds to antigen-antibody complexes. The chromogen and substrate (tetra-methylbenzidine peroxidase substrate) is added, which leads to a colour change in the presence of antigen-antibody-enzyme complexes. The reaction is stopped by adding $H_2SO_4$ after 5 – 10 minutes, and the optical density (OD) is measured spectrophotometrically. The OD reading is converted to a percentage of the positive (PP) control serum value [(mean OD of test serum/ mean OD of positive control) × 100]. The negative cut-off value is the PP value obtained with the negative control. Any samples with a PP value lower than the cut-off are regarded as negative. Test samples with values greater than the cut-off are regarded as positive *(31, 105)*.

Competitive ELISAs have been developed for the detection of group-specific antibody *(3, 70)*. A commercial kit (INgezim AHSV Compact Plus) for this purpose is available through INGENASA for the detection of IgG group specific antibodies. This assay makes use of a



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monoclonal antibody (Mab) to AHSV VP7 protein, and a recombinant VP7 antigen. In short ELISA plates are coated with the recombinant antigen, test serum samples are added and incubated. If the test sample contains VP7 specific antibody they will bind to the antigen. The plates are then washed and the VP7 peroxidase conjugated Mab is added. If no antibodies to AHSV VP7 are present in the test sera (negative sample) then the conjugate will bind to the recombinant antigen. In the case of a positive sample the conjugate will be unable to bind and therefore will be washed away in the next wash step. Following this second wash step of the plate the substrate is then added to detect the bound conjugate by means of a colorimetric reaction (OD). A decrease in the OD when compared to a control will indicate a positive result for the test sera *(3, 183)*.

*Type-Specific Serology*

Type specific antibodies can be detected using a serum neutralisation test (SNT). This test can be used to detect the presence of specific antibodies and to determine antibody titres in sera. Stock virus for each type are inoculated onto Vero cell monolayers in microtitre plates and serial dilutions of the test serum are added and incubated for four to five days until CPE is noted. The presence of specific antibodies in the test serum inhibits the production of CPE. The antibody titres are recorded as the reciprocal of the highest final dilution of serum that provided at least 50% protection of the Vero cell monolayer. A titre greater than 10 indicates that the serum is positive for those specific AHSV type antibodies. A four-fold increase in paired sample titres indicates seroconversion *(63)*. A disadvantage to this test is that as this test is type-specific, it cannot identify new types *(31)*.

It is important to note that all serological assays have a limited value for diagnosis of active infection, as many horses may die of AHS before a significant antibody response is noted *(140, 147)*.

## 1.10 AHS Controlled Area of South Africa

AHS is a controlled and notifiable disease in South Africa and South Africa has been regionalised for AHS since 1997. South African legislation defines the AHS zones constituting the AHS Controlled Area of the Western Cape Province of South Africa. The boundaries of these zones are depicted in Figure 2-1 as defined in the Animal Diseases Act (Act 35 of 1984). The rest of the Western Cape Province and South Africa is considered an AHS infected area. The boundaries of the SZ, although based on magisterial districts are essentially geographic boundaries with the Eastern boundary being the Hottentots Holland



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mountain range and the Hawekwa mountain range with the north eastern and northerly extent of the surveillance zone being the Berg river which originates in the afore mentioned mountains and flows to the west coast of South Africa entering the sea at Port Owen (Velddrift) *(157)*. The creation of the zones in these areas is based on the epidemiology of AHS in South Africa where the north eastern parts of the country are considered endemic with annual spread of infection in a south westerly direction which is halted by first frost, generally in May each year.

***AHS Surveillance in the AHS Controlled area***

According to the OIE code *(180)* an AHS case is defined as:

1. *AHSV has been isolated and identified by means of virus isolation, OR*
2. *An agent identification test (RT-qPCR) is positive and there are clinical signs consistent with AHS or there is epidemiological evidence consistent with AHS, OR*
3. *There is seroconversion not due to vaccination with clinical signs consistent with AHS or there is epidemiological evidence consistent with AHS*

As surveillance involves the detection of disease or infection *(180)* the test methods used should be carefully considered and the diagnostic specificity and sensitivity of the tests should be known.

Surveillance deals not only with the occurrence of clinical signs caused by AHSV, but also with the evidence of infection in the absence of clinical signs *(180)*. According to the OIE code an AHS free country or zone should include an early warning system for reporting of suspect cases, these cases would then require extensive follow-up and investigation to confirm or exclude AHS as a cause.

The EU monitoring requirements for export from the AHS free zone in South Africa according to the EU Commission Decision of 2008 (2008/698/EC) include monthly sero-epidemiological surveillance of at least 60 identified unvaccinated sentinel horses spread over the AHS free and surveillance zones. All cases of equine mortality in the free area due to a suspected infectious disease are examined by official equine necropsy and AHS is either confirmed or excluded by appropriate testing.



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### *Passive Clinical Surveillance*

This technique involves the reporting of all suspect AHS cases to the local state veterinary authority. This is a requirement by law in South African *(157)*. A suspect case will only be considered confirmed if it fulfils at least one of the criteria described in the case definition above. One of the complicating factors in this method of surveillance is the fact that an AHSV-LAV can cause disease, and can also potentially be transmitted. Currently whole genome sequencing is the only method available to definitively differentiate vaccine-induced positive cases from natural infection making interpretation of result from screening testing methods in previously vaccinated animals extremely difficult.

### *Sentinel Surveillance*

The use of sentinel animals is a form of targeted surveillance in a prospective study design. Normally sentinel animals should be unexposed animals that are not vaccinated and managed at fixed locations. One of the problems with this method is that a high level of vaccination coverage in the population of the Controlled area means that serological surveillance is difficult. The shortage of true Sentinel animals that have never been vaccinated in the AHS free and surveillance zones has also made this surveillance strategy problematic. In the AHS free and surveillance zones the Sentinel Programme has been adjusted to account for these difficulties. The programme currently implemented involves monthly sampling of horses, donkeys or mules year round. The equines must be unvaccinated, or not have been vaccinated with AHSV-LAV for at least the preceding 2 years. The testing procedure is primarily based on the demonstration of absence of circulating virus by group specific RT-qPCR. The sample size and geographical distribution is based on an intensive and on-going census programme within the AHS free and surveillance zones. Serological testing by means of an iELISA *(105)* is also applied to those animals within this group that are true, unvaccinated sentinels, to show continued sero-negativity and lack of seroconversion, providing added confidence to the current surveillance strategy. This surveillance strategy is currently implemented as a collaborative effort between the Western Cape State Veterinary Services and the Equine Research Centre, University of Pretoria.

A Sentinel Foal surveillance programme has also been proposed, but to date not implemented. This programme would involve the monthly sampling of unvaccinated foals during the high risk season. In this case the testing would be primarily based on evidence of



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seroconversion, with sample storage and subsequent testing by PCR for agent identification in the case of evidence of seroconversion.

***Wildlife Surveillance***

Wildlife surveillance within the AHS SZ currently comprises opportunistic sampling of zebra with serological testing and group specific PCR testing for agent identification.

***Vector Surveillance***

Vector surveillance is confined to the export quarantine station within the AHS free zone, in order to monitor trends in vector population and effectiveness of the vector proof system for the quarantine facility.



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# CHAPTER 2

## The 2011 outbreak of African horse sickness in the African horse sickness controlled area in South Africa

### 2.1 Abstract

African horse sickness (AHS) is a controlled animal disease in South Africa and outbreaks within the AHS controlled area in the Western Cape Province have a significant impact on affected properties as a result of the high mortality rates experienced as well as on the exportation of live horses from the AHS free zone in metropolitan Cape Town. An outbreak of AHS type one occurred in the surveillance zone of the AHS controlled area of the Western Cape during the summer of 2011. The epicentre of the outbreak was the town of Mare in the magisterial district of Malmesbury, and the outbreak was confined to a defined containment zone within this area through movement control of all equips and a blanket vaccination campaign. A total of 73 cases of AHS were confirmed during this outbreak which included four confirmed subclinical cases. The morbidity rate for the outbreak was 16% with a mortality rate of 14% and a case fatality rate of 88%. Outbreak disease surveillance relied on agent identification using PCR based assays which is novel for an AHS outbreak in South Africa. The source of this outbreak was not confirmed at the time although it was believed to be associated with an illegal movement of an infected animal into the Mamre area at the time. The detailed description of the outbreak provides a sound scientific basis to assist decision making in future AHS outbreaks in the AHS controlled area of South Africa and in countries where AHS is an exotic or emerging disease.

### 2.2 Introduction

African horse sickness (AHS) is a vector-borne life threatening disease of equids caused by African horse sickness virus (AHSV), a member of the genus *Orbivirus* in the family *Reoviridae*. This virus causes vascular injury that can result in four forms of disease: the pulmonary, cardiac, mixed or horse sickness fever forms *(30)*. The virus is transmitted to horses by midges (*Culicoides spp.*) and the disease is most prevalent in areas where these vectors are most abundant. Outbreaks in South Africa generally occur during late summer in the summer rainfall areas of the country *(30)*. AHS is a controlled disease in South Africa *(157)* and is a World Organisation for Animal Health (OIE) listed disease as it causes high mortality rates and has the potential for rapid spread *(114)*. AHS is endemic to sub-Saharan Africa but the disease has occurred sporadically outside of this region *(116)*. An example of



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this is the outbreak of AHS in Spain in the 1960's and 1980's where hundreds of horses succumbed to the disease while hundreds of thousands were vaccinated during its control *(140)*.

The area around the Cape of Good Hope in South Africa has historically been free from AHS *(30)*, with outbreaks as a result of the introduction of AHSV positive horses from other provinces. On this basis a protocol was submitted to the European Community (now the European Union (EU)) proposing the establishment of an AHS free zone in the Cape Peninsula, from which the export of horses could resume provided certain conditions were met *(22)*. This proposal was accepted by the EU in 1997. An AHS controlled area was established in the Western Cape Province of South Africa *(157)* in 1997 consisting of the Metropolitan Cape Town AHS free zone, the AHS surveillance zone and the AHS protection zone (section B of Figure 2-1). Since implementation of this regionalisation two other AHS outbreaks occurred in the Stellenbosch district *(155)* within the AHS surveillance zone. These outbreaks were as a result of AHSV7 in 1999 and AHSV1 in 2004 and the equine deaths associated with these outbreaks totalled 32 and 16 respectively. In February 2011 another outbreak of AHS occurred within the AHS surveillance zone in horses resident in the town of Mamre in the Malmesbury magisterial district. This resulted in the suspension of horse exports directly from South Africa to the EU and other countries. The aim of this study was to provide a descriptive evaluation of the events and control measures employed for this outbreak of AHS.

## 2.3 Materials and methods

### *The outbreak*

On 26 February 2011 State Veterinary (SV) services, Malmesbury, was requested by a private veterinarian from the Malmesbury district to assist in the necropsy of a horse that had died that day in the town Mamre in the Western Cape Province of South Africa. The private veterinarian had seen the horse earlier in the day and it had exhibited clinical signs suggestive of infection with AHSV. Blood and tissue samples were collected for laboratory investigation. On the same day the SV sampled a dead horse found next to the road in Mamre. On 02 March another horse was reported dead by an owner from Mamre and a necropsy with sampling was performed. Results of quantitative real time polymerase chain reaction (RT-qPCR) assays were also received showing that the samples from the first two mortalities were positive for AHSV. The Department of Agriculture, Forestry and Fisheries (DAFF) were notified immediately and the implementation of control measures and plans to



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attempt to contain the outbreak were initiated by the Directorate of Veterinary Services of the Western Cape Province.

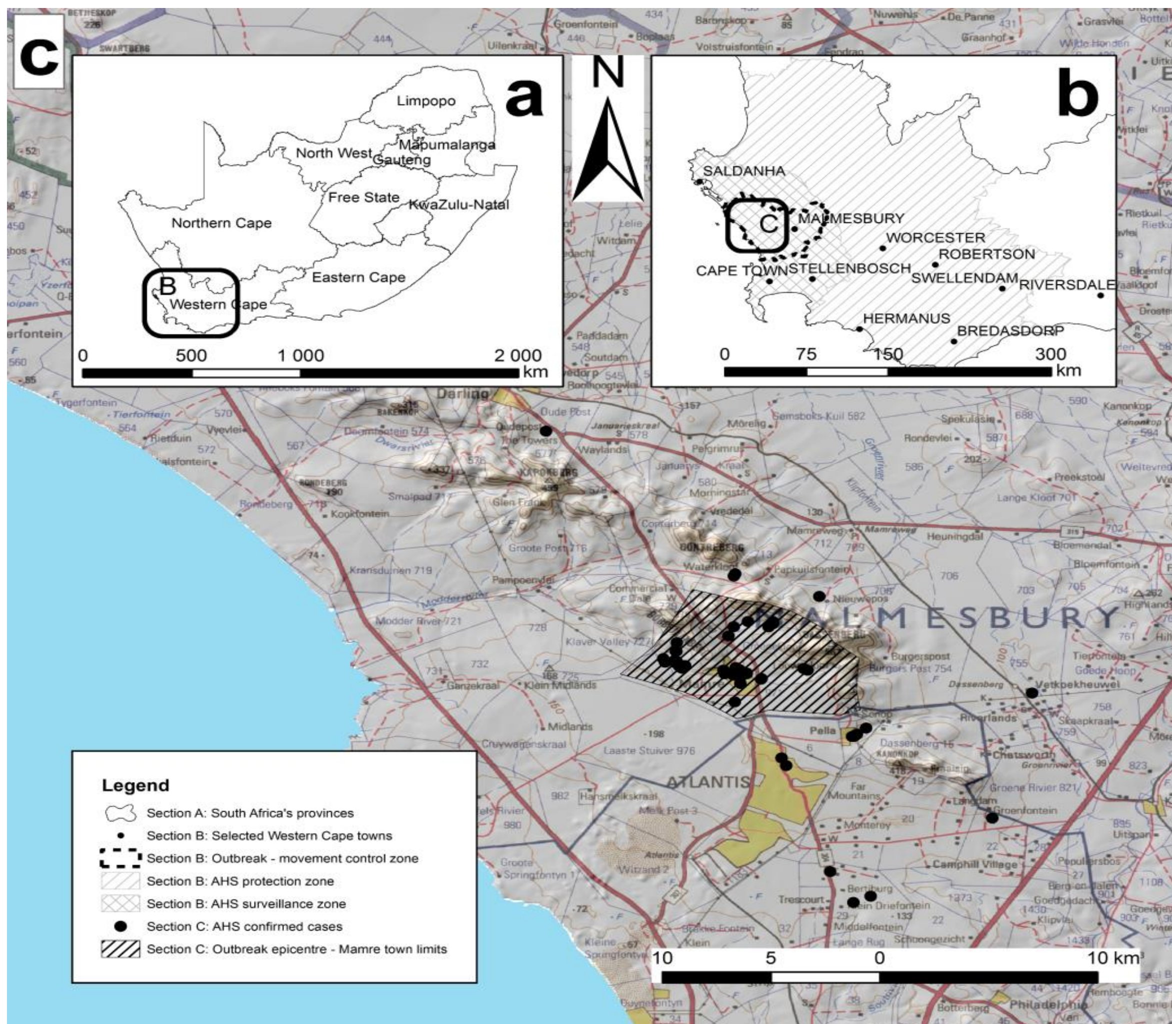


**Figure 2-1: A map indicating the extent of the AHS outbreak in the Western Cape. Section A shows the geographical extent of South Africa with the Provincial boundaries as well as the extent frame of section B (labelled as such). Section B shows two of the AHS control zones within the Western Cape Province, namely the Protection zone which is an area of compulsory vaccination for domestic equids against AHS; and the Surveillance zone which is where vaccination is only performed after permission from the Provincial Director of Veterinary Services is obtained. Also in Section B is the extent of the Malmesbury magisterial district, the boundaries of which were used to delineate the movement restriction zone implemented during the outbreak. Section C shows the extent of the outbreak by individual confirmed cases (n=73) within the Malmesbury district as well as the extent of the Mamre town limits which was the epicentre of the outbreak and where the majority (n= 57) of cases occurred.**

A movement ban was immediately instituted by means of a press release on 03 March 2011 that limited movement of horses into, within and out of the Malmesbury magisterial district. These movement restrictions remained in place until 09 June 2011, one month after the last confirmed case (03 May 2011) of AHS within the containment zone.



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Advice was given to horse owners to stable their horses from two hours before sunset until two hours after dawn to decrease the risk of vector contact with the horses. Owners were also advised to apply insect repellent to their horses on a daily basis.

Ring vaccination was initiated on 09 March 2011 within the immediate vicinity of the initial cases in Mamre. The vaccine used was a live attenuated vaccine (LAV) produced by Onderstepoort Biological Products (OBP) which is presented as two separate injections with different AHS virus combinations represented in each injection. The outbreak strain was identified as AHSV1 and so the relevant vaccine (OBP comb1) was used during the primary outbreak response vaccinations to provide protection against this type. Private veterinarians within the area were authorised to vaccinate clients' horses resident within the Malmesbury magisterial district provided they kept detailed records of these vaccinations. This authorisation for private veterinarians to perform emergency vaccination within the Malmesbury magisterial district was rescinded after the conclusion of the outbreak. The ring vaccination provided an opportunity for a concurrent equine census to be taken from the outbreak area. The population at risk used in the denominator for the estimations of disease morbidity and mortality were calculated from these data.

***Sample collection and laboratory testing***

Samples collected from dead horses for laboratory testing included lung and spleen samples. When carcasses were badly decomposed a sample of body fluid was placed in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes or a piece of tissue was collected. When a veterinarian was present EDTA blood samples were collected by jugular venipuncture from each horse presented to outbreak response personnel prior to vaccination.

Blood and organ samples were subjected to testing using either a RT-qPCR *(61, 137)* or a hemi-nested PCR (hnPCR) assay *(24)*. In the case of the RT-qPCR the quantification cycle value (Cq) is the number of cycles taken for fluorescence in the sample to exceed a threshold of 0.1. For the purposes of this outbreak, RT-qPCR results were classified as positive if the fluorescence exceeded the threshold of 0.1 within a maximum of 30 cycles.

In order to rapidly establish the type of AHSV involved in the outbreak, partial, direct sequencing of the S10 gene (coding for the non-structural protein NS3) and the L2 gene (coding for the outer capsid protein VP2) was performed using the samples collected from the first confirmed case of AHS of the outbreak. Nucleic acid was extracted using a MagMAX™ Express Magnetic Particle Processor and a MagMAX™-96 Total RNA Isolation



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Kit (Lifetech), according to the manufacturer's instructions. Sequencing of S10 was performed as described previously *(138)*. Primers for sequencing L2 were designed from AHSV sequences available on Genbank. L2 was amplified in approximately 600 nucleotides (nt), overlapping fragments and the sequences assembled using the Staden package. MEGA4 was used to construct a bootstrapped (1 000 replications) neighbour-joining phylogenetic tree of approximately 1 000 nucleotides of the 5'-end of the AHSV L2 gene.

Virus isolation and serotyping by the plaque inhibition neutralisation test were performed as described previously *(138)*.

***Outbreak case definition***

The AHS case definition used by the Directorate of Veterinary Services, Western Cape Province at the beginning of the outbreak was clinical and/or post-mortem signs indicative of AHS with laboratory confirmation by PCR and/or virus isolation assays. Initially, only horses which showed clinical and/or post mortem signs indicative of AHS were included as suspect cases. This was subsequently modified when it became evident that some horses which did not show clinical signs indicative of infection with AHSV tested positive on PCR and in some cases AHSV type one was isolated from samples collected from these horses. A case definition code system was instituted in order to facilitate processing of results (Table 2-1).

**Table 2-1: The outbreak case definition codes categorised according to the clinical signs and laboratory results of each case, and the number of cases in each category.**

| Case Definition | Total of cases per case definition | Code | Sub definition | Number of cases per sub definition |
|---|---|---|---|---|
| Negative | 172 | N1 | Death or suspect AHS clinical and/or PM signs with confirmation of another cause of disease and confirmed negative AHS laboratory results | 14 |
| | | N2 | Routine testing for surveillance with negative AHS laboratory results | 158 |
| Positive | 73 | P1 | Clinical signs and/or PM signs of AHS with laboratory PCR and/or VI positive result | 69 |
| | | P2 | PCR and VI AHS positive result with no accompanying clinical or PM signs | 4 |
| Suspect | 16 | S1 | Clinical signs and/or PM signs of AHS with no laboratory positive confirmation | 4 |
| | | S2 | No clinical or PM signs of AHS with a positive PCR result and VI negative | 12 |

Subclinical cases were defined as an animal that was found to be RT-qPCR (n=14) or hnPCR (n=1) positive, with or without positive AHS viral isolation (VI) results, but showing no discernible clinical signs of AHS. AHSV type 1 was isolated from four of these cases which were subsequently classified as confirmed cases.



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The case definition for an AHS case according to the OIE *(180)* is:

*'AHSV has been isolated and identified from an equid or a product derived from that equid; or*

*viral antigen or viral RNA specific to one or more of the types of AHSV has been identified in samples from one or more equids showing clinical signs consistent with AHS, or epidemiologically linked to a suspected or confirmed case; or*

*serological evidence of active infection with AHSV by detection of seroconversion with production of antibodies to structural or non-structural proteins of AHSV that are not a consequence of vaccination have been identified in one or more equids that either show clinical signs consistent with AHS, or epidemiologically linked to a suspected or confirmed case.'*

According to this definition all the suspect horses falling into the S2 (n=12) case definition category should also have been considered as confirmed AHS cases due to the animals being epidemiologically linked to other confirmed cases, and having a positive molecular diagnostic result. This should be taken into consideration in future outbreaks as well as reporting of cases from the infected part of the country.

***Carcass disposal***

Horse carcasses on which post mortems were performed at the Stellenbosch provincial veterinary laboratory were disposed of by the laboratory. Dead horses that were not transported to the laboratory for post mortem were sampled in situ and transported to the Vissershok waste disposal site which is registered for this purpose. There were exceptional cases where disposal was not possible and this was when carcasses were too decomposed to move or where attempts by local residents had been made to burn or bury them locally.

***Ethical considerations***

Samples collected during this outbreak were done under State authorisation using standard veterinary procedures and according to disease detection and surveillance protocols.



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## 2.4 Observations

### *Quantification and spread of the outbreak*

The various epidemiological variables shown in Table 2-2 have been separated into the outbreak as a whole and into the outbreak epicentre. The outbreak epicentre was the area within the Mamre town limits where most cases occurred and it is approximately 3900 Ha in size. Outlying cases (Figure 2-1) extended as far as Darling to the north-west (15 kilometres (km)) Riverlands in the east (12 km), Groenfontein to the south-east (13 km) and just south-east of Atlantis to the south (13 km).

**Table 2-2: Various epidemiological variables measured during the outbreak. Equines evaluated include all equines which were surveyed using RT-qPCR testing by the Equine Research Centre (UP). Population at risk is based on the census performed in the outbreak area by the veterinary services of the Western Cape. 95% confidence intervals have been included in brackets where applicable.**

| Aspect measured | Outbreak area category | |
|---|---|---|
| | Epicentre (Mamre town limits) | Entire outbreak |
| Estimated population at risk based on census data | 319 | 447 |
| Animals evaluated: clinically, post mortem or laboratory tested | 186 | 261 |
| Total positive cases | 57 | 73 |
| Total AHS confirmed deaths | 50 | 64 |
| Morbidity rate | 0.18 (0.14–0.22) | 0.16 (0.13–0.20) |
| Mortality rate | 0.16 (0.12–0.20) | 0.14 (0.11–0.18) |
| Case fatality rate | 0.88 (0.76–0.94) | 0.88 (0.78–0.94) |
| Total confirmed sub clinical cases | 4 | 4 |
| Total suspected sub clinical cases | 9 | 11 |

The confirmed AHS cases totalled 73 horses. The incidence of AHS during the outbreak period in the epicentre was 0.18 (95% Conf. 0.14 – 0.22) compared to an incidence of 0.16 (95% Conf. 0.13 – 0.20) for the outbreak as a whole. The total deaths recorded during the outbreak (both AHS confirmed (n=64) and suspect cases (n=6)) was 70 horses. This relates to an AHS specific mortality rate of 0.16 (95% Conf. 0.12 – 0.20) in the epicentre and 0.14 (95% Conf. 0.11 – 0.18) for the outbreak as a whole. The majority of these deaths impacted indigent owners. The case fatality rate was 0.88 both within the outbreak epicentre and for the outbreak as a whole.

There were a number of subclinical cases recorded in this outbreak, both suspect (n=11) and confirmed (n=4), which is also a unique feature for an outbreak in the surveillance zone, but has been recently described in endemic areas *(176)*. Ten of the 11 suspect subclinical cases were RT-qPCR positive, some for an extended period of time, but isolation of virus



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from these blood samples proved unsuccessful. Of the 11 suspect cases, 9 were sampled prior to vaccination, so the positive RT-qPCR result was most likely as a result of field infection and not vaccination. The other two were sampled between one and two weeks after vaccination, so the positive result may have been due to vaccination or field infection. There was one remaining suspect case that was not classified as subclinical as the horse died. However, although the horse was found to be RT-qPCR positive, the PM findings were not typical of AHS, the sample was found to be negative on VI and the horse's vaccination history was unknown. It was therefore decided to classify this horse as suspect rather than confirmed. One other limitation within the subclinical case category is that the number of suspect subclinical cases is dependent on the specificity of the PCR assay used in their definition. The median specificity of the RT-qPCR used in the outbreak (defining 10 of the 11 suspect cases) is described as being greater than 0.992 *(61)*.

A total of 447 equids were vaccinated against AHS by state veterinary authorities in the outbreak area, with the majority being in early March 2011, approximately one week before the outbreak peaked (Figure 2-2). Reports from the Malmesbury district show that a further 189 horses were vaccinated by private veterinarians during the outbreak period in the surrounding district. This brought the total of vaccinated equines during the outbreak period to 636.



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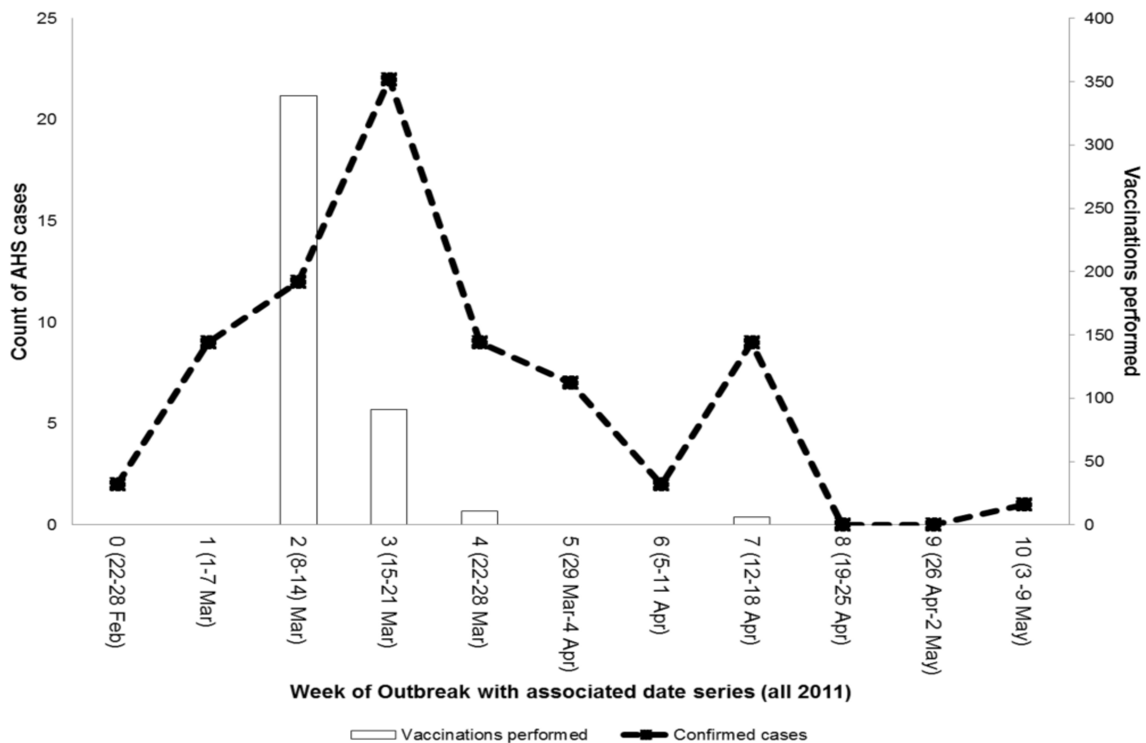


**Figure 2-2: The epidemic curve (dashed line) of the outbreak depicting only AHS confirmed cases (n=73). The peak of cases occurred during the fourth week (including week zero) of the outbreak with 22 cases occurring. The curve drops off sharply with a small secondary peak during the eighth week of the outbreak. The vaccinations performed as a result of the outbreak (private veterinarian vaccinations not included; totals per week shown as bars with the totals on the right axis) show that the peak vaccination occurred in the week before the peak in confirmed cases.**

### *Virus identification*

A sequence of about 320 nt long at the 5' end of AHSV S10 was obtained. The sequence shared the highest identity with two AHSV1 isolates; 99.4% with E03404 (GenBank accession number EU433546.1) and 99.6% with Jane 1 (EU433410). Sequencing of approximately 1 000 nucleotides of the 5'-end of the AHSV L2 showed that the isolate was AHSV1. Alignment of the sequence with other AHSV VP2 sequences available on GenBank showed a 99.8 – 99.9% identity with other AHSV type one isolates (Figure 2-3).



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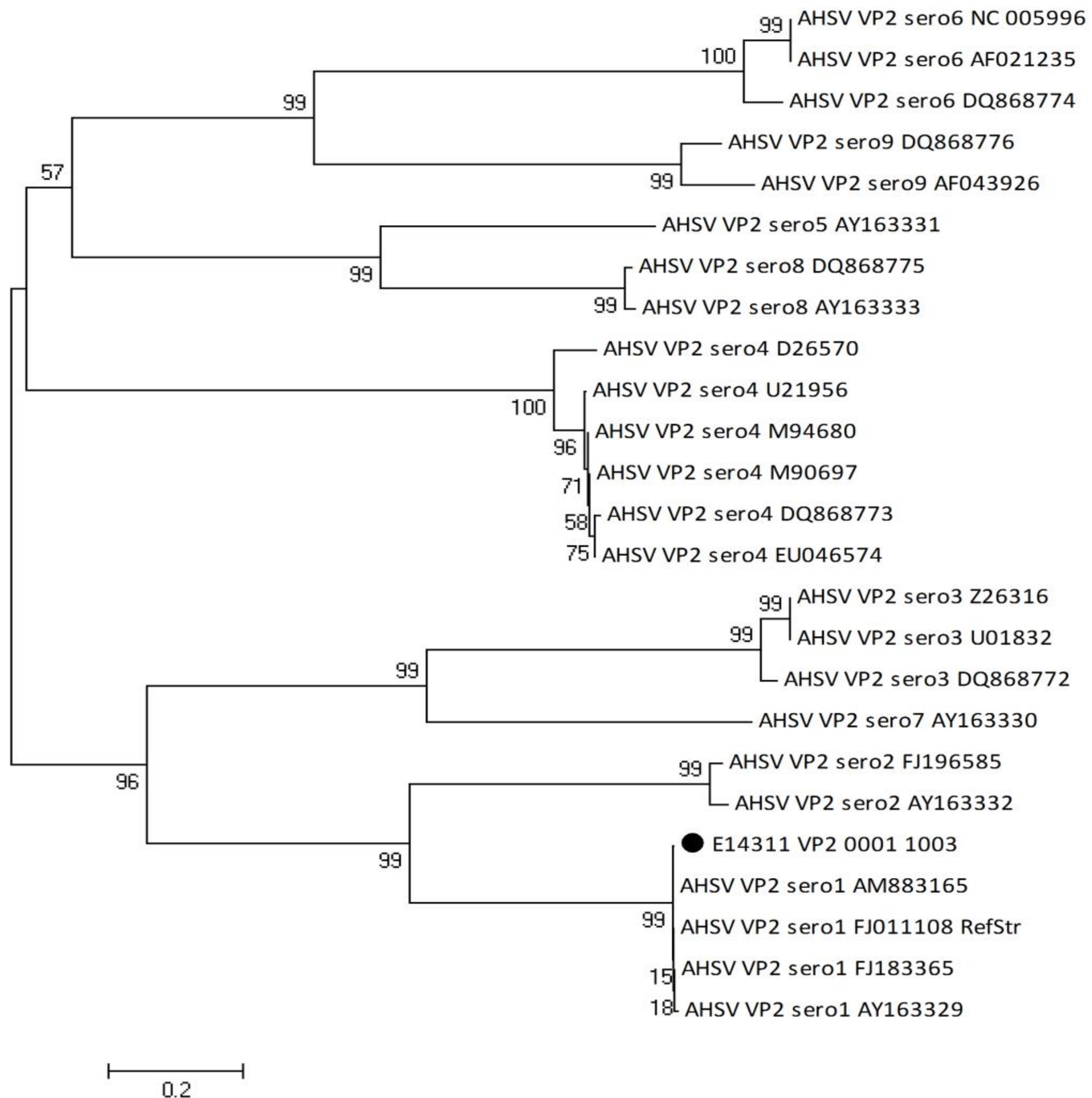


**Figure 2-3: A neighbour-joining phylogenetic tree showed that the outbreak isolate (E14311 VP2 0001 1003) grouped with other AHSV type one isolates.**

## 2.5 Discussion

***Spread and source of disease***

The demographics within the outbreak environment meant that it was initially difficult to prevent the movement of infected animals within the Mamre area and immediate surrounds, as there were no formal horse societies to advise and pressurise owners, and no incentive not to move horses as per normal. It was found that personal communication with each



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individual horse owner in Mamre and surrounding areas was necessary in order to explain the reasons for movement restrictions. This was one of the biggest challenges in control of this outbreak compared to past outbreaks of this disease which occurred within wealthier socioeconomic areas where formal stabling and management existed. The movement control strategies into, out of and through the movement restriction zone were effective in preventing formal movements via vehicles, which was easier to control as there was cooperation from traffic officials and reporting from other concerned horse owners.

The source of the outbreak could not be confirmed at the time, although investigations by the SV did show that there were suspected illegal movements of horses into the AHS surveillance zone. The partial sequence of the S10 gene of the outbreak virus was 98-99% identical with other AHSV1 viruses including the Jane reference strain and field isolates from Mpumalanga, Gauteng and from the Stellenbosch area of the AHS surveillance zone. Further phylogenetic studies of these AHSV1 viruses and AHSV1 viruses isolated in South Africa prior to and following the 2004 AHS surveillance zone outbreak may provide data from which the most likely source of the outbreak virus could be identified. Such studies would also be necessary to confirm that the outbreak viruses involved in 2004 and 2011 were sufficiently different to be associated with separate introductions and were not due to re-emergence of the 2004 outbreak virus in 2011. As the prevailing wind direction on the Cape peninsula in the summer months originates from the south or south-east and the only AHS cases reported in other parts of South Africa prior to the outbreak in the surveillance zone in 2011 were in excess of 800km from the outbreak in a north-easterly direction it is highly unlikely that wind borne dissemination of infected *Culicoides* were responsible for initiating this outbreak.

***Outbreak surveillance strategy***

A unique feature with regards to the surveillance approach to this outbreak was the surveillance methods employed in the apparently healthy equine population. This ensured that active AHSV nucleic acid detection surveillance on an almost census level sampling frame was accomplished and this provided a dataset from which accurate determination of epidemiological variables could be made as testing results improved the accuracy of confirmed negative cases in the population at risk (Table 2-2). Serological surveillance techniques have been utilised in previous outbreaks but there is always the aspect to consider of baseline vaccine antibody levels present in the horse at the time of sampling and invariably paired serum samples are a necessity. With PCR techniques, and in particular in future with type specific real time PCR, one sample per horse examined or included in the



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census, in the initial stages of an outbreak will assist greatly in determining the initial extent and magnitude of the outbreak. Making use of a nucleic acid detecting test, in this case PCR on EDTA blood samples, for surveillance during an outbreak does also pose its own set of challenges. The window of opportunity for detecting nucleic acid is reliant on the period these molecules are at detectable levels within the tested substrate. Also, as in this outbreak, once the blanket vaccination of equines in the area took place, the value of PCR surveillance diminished dramatically as one would experience if serological surveillance took place, as in both cases vaccination versus outbreak strain aetiology cannot easily be established post vaccination. PCR or serological testing making use of a DIVA (differentiating infected from vaccinated individuals) assay will greatly assist in any outbreak situation and AHS outbreaks would not be an exception.

The subclinical cases found in this outbreak, although substantially fewer than the clinical cases, are still a cause for concern when considering spread of disease. This is of further concern when considering that these cases, without the aid of molecular surveillance methods such as was employed using the RT-qPCR during this outbreak, are unlikely to be diagnosed in field conditions as the relevant samples would generally not be taken as the horse would not show clinical signs. An evaluation of all positive (P1 and P2 definitions) and suspect (S2 definition) cases where PCR and VI results were available showed that only 66% (n=54) of the positive PCR results had collaborating positive VI results. Of the total 15 suspect subclinical cases only four (27%) had positive virus isolation results again showing the value of the more sensitive PCR in an outbreak situation.

***Vector surveillance***

By far the majority of captured *Culicoides spp.* taken at the southern collection site had *C. imicola* as the dominant species, which was to be expected. However, the more dominant species in collections in Mamre was *C. subschultzei* with *C. imicola* of secondary importance. *C subschultzei* has been reported in the surveillance zone in the past; however they have never been reported in large numbers and have never been reported as the dominant species in South Africa *(168)*. The importance of *C. subschultzei* in the transmission of AHS has yet to be determined.

***Vaccination effect***

A dramatic decrease in the number of cases occurring per week was observed within two weeks after the majority of the equine population in Mamre had been vaccinated (Figure 2-



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2). The majority of equids vaccinated (n=289) were within the Mamre town limits. This accounted for 64.6% of equines vaccinated in the outbreak zone and this covered 90% of the equines within Mamre.

As AHS is a vector borne disease one would normally expect (if no counter measures are taken) a propagating epidemic curve with multiple case peaks during an outbreak. Except for a very small peak at the end of April the outbreak in Mamre only showed one peak in disease cases. This is most likely due to vaccination in the Mamre area and particularly in the surrounding areas of Pella and Atlantis which prevented significant seeding of the outbreak.

***Impact of the outbreak***

The direct costs of this outbreak were borne by many different industries and state departments. The lowest cost estimate for the outbreak totalled R850 000 and the majority of this cost was due to laboratory testing of samples. The equine industry paid for much of the monetary expenses associated with the outbreak, and the role-players involved included: Equine Research Centre (University of Pretoria); Racing South Africa; Cape Breeders Association and the Acorn Group of Companies. State departments involved included: Western Cape Department of Agriculture - Veterinary Services Animal Health; City of Cape Town Disaster Management and the Stellenbosch Provincial Veterinary Laboratory.

At the time of the outbreak, South Africa was exporting on average 200 horses per annum. The revenue loss to industry stakeholders directly involved in the logistics of exporting horses is estimated at R20 million per annum. There was also loss of foreign investment as a result of a decrease in direct exports which was estimated at R200 million per annum. This was partly (by 33%) offset by importers utilising the alternative shipping routes via Mauritius. (P Gibson, Racing South Africa, pers. comm., 2012).

Seventy horses in total (64 confirmed as AHS) died during the 2011 AHS outbreak in one of the poorer communities in the Western Cape. Apart from the tradable value of these horses the direct and indirect cost as a result of the death of these animals is difficult to determine. Direct costs include the loss of a working animal - in terms of transport and for herding livestock. Indirect costs include all products and services related to the maintenance of the horses in this community. Emotional costs and the loss of breeding stock are also important factors to consider as horses are considered as part of the community’s heritage.



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## 2.6 Conclusion

The response to this outbreak highlighted the importance of partnerships in the control of diseases such as AHS. Private-public partnerships between members of equine industries and the State Veterinary service ensured a rapid control response and the State was able to start vaccinations immediately with the AHS vaccine donated by the Equine Research Centre, and the personnel of the different bodies were able to work together in an efficient manner. This also helped in building a relationship with the community of Mamre, as all parties involved were seen to have the same objectives and the best interests of the community at heart.

The vaccination requirements described by the movement control protocol of horses *(16)* between the various AHS control zones within South Africa is primarily aimed at preventing infected horses entering the AHS surveillance zone, which in turn decreases the risk of AHS infection within the AHS free zone. The various equestrian societies that rely on movements of horses for their events must continue to ensure that horses participating have undergone the correct procedures when moving between the various AHS control zones to minimise the risk of future incursions.



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# CHAPTER 3

## Development of three triplex real-time reverse transcription PCR assays for the qualitative molecular typing of the nine types of African horse sickness virus

### 3.1 Abstract

Blood samples collected as part of routine diagnostic investigations from South African horses with clinical signs suggestive of African horse sickness (AHS) were subjected to analysis with an AHS virus (AHSV) group specific reverse transcription quantitative polymerase chain reaction (AHSV RT-qPCR) assay and virus isolation (VI) with subsequent serotyping by plaque inhibition (PI) assays using AHSV type-specific antisera. Blood samples that tested positive by AHSV RT-qPCR were then selected for analysis using AHSV type specific RT-qPCR (AHSV TS RT-qPCR) assays. The TS RT-qPCR assays were evaluated using both historic stocks of the South African reference strains of each of the 9 AHSV types, as well as recently derived stocks of these same viruses. Of the 503 horse blood samples tested, 156 were positive by both AHSV RT-qPCR and VI assays, whereas 135 samples that were VI negative were positive by AHSV RT-qPCR assay. The virus isolates made from the various blood samples included all 9 AHSV types, and there was 100% agreement between the results of conventional serotyping of individual virus isolates by PI assay and AHSV TS RT-qPCR typing results. Results of the current study confirm that the AHSV TS RT-qPCR assays for the identification of individual AHSV types are applicable and practicable and therefore are potentially highly useful and appropriate for virus typing in AHS outbreak situations in endemic or sporadic incursion areas, which can be crucial in determining appropriate and timely vaccination and control strategies.

### 3.2 Introduction

African horse sickness (AHS) is an arboviral disease of horses that is endemic throughout much of sub-Saharan Africa, but significant incursions have occurred previously into North Africa, the Iberian Peninsula, the Middle East and the Indian subcontinent *(30, 60, 99)*. Nine types of AHS virus (AHSV) have been described *(82, 109)*. The genome of AHSV consists of 10 double stranded RNA segments, encoding 7 structural (VP1 to VP7) and 4 non-structural (NS1, NS2, NS3/NS3A and NS4) proteins *(57, 144, 188)*. Genome segment 7 (S7) encodes the inner capsid protein VP7, which is highly conserved among the 9 AHSV types *(137)* and is the basis for several antigen *(69, 94)*, antibody *(70, 91, 105, 174)*, and nucleic acid based



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diagnostic assays *(1, 48, 61, 137, 186)*. An outbreak of AHS in a naïve horse population can be devastating, with a cumulative mortality rate of up to 95% *(30, 60, 114)*.

A tentative diagnosis of AHS can be made on the basis of clinical signs and post mortem findings. Clinical signs considered indicative of AHS include those of pyrexia, supraorbital fossa swelling, subcutaneous oedema and respiratory distress due to pulmonary oedema *(60)*. These symptoms should be confirmed by laboratory diagnosis, as differential diagnoses such as Equine encephalosis virus infection must be ruled out. This has been done historically using virus isolation (VI), with subsequent determination of the type of individual virus isolates by virus neutralisation assays *(30, 60)*. This process is time-consuming, labour-intensive, and expensive. Several AHSV group-specific reverse transcription polymerase chain reaction (RT-PCR) assays have been developed recently for the diagnosis of AHS *(8, 10, 61, 146, 158)*. Advantages of AHSV RT-PCR assays are that they have the potential to be rapid, sensitive and versatile, and can supplement existing traditional virus identification methods. AHSV RT-PCR assays can also be applied to samples that do not contain infectious virus, or have very low viral titres, making them ideal screening tests *(137)*. However, samples that are positive by RT-PCR, but negative by VI are common, especially amongst horses previously immunized against AHS with live-attenuated vaccine *(61, 176)*. Determination of the type of AHSV contained in such samples currently requires further evaluation, with additional testing.

Determination of AHSV type is important in epidemiological studies and in order to quickly implement appropriate vaccination and control strategies. For example, outbreaks of AHS among horses in the AHS-controlled area in the Western Cape region of South Africa have highlighted the need for rapid and specific determination of the type of the incurring virus. Traditional VI and serotyping was used previously to identify the AHSV-1 type involved in an outbreak of AHS in the region in 2004 *(155)*, whereas the AHSV-1 that was responsible for the outbreak in 2011 was determined by sequence analysis of the AHSV type-specific L2 (VP2) gene of the causative virus contained in the blood of an affected horse. This latter process took six days to complete before vaccination could be instituted *(56)*. AHSV type-specific real-time RT-PCR (TS RT-qPCR) assays offer the potential for more rapid determination of the AHSV type involved in such outbreaks. AHSV TS assays that target the portion of the L2 gene encoding the major neutralisation determinants of AHSV *(27, 86, 133, 173)* are well suited for accurate, rapid type determination. Direct TS RT-PCR assays have been developed *(146)*; however these assays are cumbersome and time consuming as they require the use of gels for assay confirmation. Direct TS RT-PCR assays also increase the risk of contamination and false positive results. Koekemoer developed an AHSV TS RT-



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qPCR assay that utilizes a sensor probe and an anchor probe on two different channels, followed by melt curve analysis to differentiate the various AHSV types *(90)*. Although these assays accurately confirmed the type of historic prototype South African strains of AHSV, they showed considerable variation in determination of the AHSV type of field strains. More recently, TS RT-qPCR assays for the detection and typing of AHSV have been developed and described *(10)*.

The objective of the current study was to develop and characterize the applicability and practicability *(26)* of AHSV TS RT-qPCR assays in a multiplex format (3 triplex assays) for the rapid molecular typing of samples determined to be positive for AHSV using a group specific RT-qPCR of documented diagnostic accuracy. Such assays will facilitate in the rapid determination of the virus type in field outbreaks of AHS thereby facilitating implementation of appropriate vaccination and control strategies in endemic areas such as South Africa. The applicability and practicability of these assays was evaluated when the assays were applied to nucleic acid extracted from blood samples collected from field cases of suspected AHS.

## 3.3 Materials and Methods

### *Reference Strains of AHSV*

South African reference strains of the 9 AHSV types were obtained in 1995 from the World Organization for Animal Health (OIE) AHS Reference Laboratory at the Onderstepoort Veterinary Institute (OVI). New reference strains of the 9 AHSV types were obtained in 2014 from the same laboratory. Source data were not available for the 1995 reference strains. Details of the 2014 reference strains are provided in Table 3-1. The relationship between the two sets of reference strains is unknown. Reconstituted freeze-dried reference viruses obtained in both 1995 and 2014 were extracted and evaluated using the group specific AHSV RT-qPCR assay as previously described *(61)*, followed by analysis with the AHSV TS RT-qPCR assays described below.



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**Table 3-1: Information on the origin of the African horse sickness virus reference strains (2014); showing the name, isolate name and country of isolation with passage history and GenBank accession numbers for genes encoding VP2 of each virus.**

| Name | Isolate | Country | Passage History | GenBank Accession No. | |
|---|---|---|---|---|---|
| | | | | ARC-OVI | ERC |
| **AHSV1** | HS29/62 | South Africa | Mouse #2, BHK #2 | KP939376 | KT030571 |
| **AHSV2** | HS82/61 | South Africa | Mouse #2, Vero #1, BHK #2 | KP939429 | KT030581 |
| **AHSV3** | HS13/63 | South Africa | Mouse #3, Vero #1, BHK #2 | KP939488 | KT030591 |
| **AHSV4** | HS32/62 | Zimbabwe | Vero #1, BHK #2 | KP939584 | KT030601 |
| **AHSV5** | HS30/62 | South Africa | Mouse #2, BHK #2 | KP939711 | KT030611 |
| **AHSV6** | HS39/63 | South Africa | Vero #1, BHK #2 | Not done | KT030621 |
| **AHSV7** | HS31/62 | South Africa | Mouse #1, Vero #2, BHK #2 | KP939937 | KT030641 |
| **AHSV8** | HS10/62 | Kenya | Mouse #1, Vero #1, BHK #2 | KP940010 | KT030651 |
| **AHSV9** | HS90/61 | Chad | Mouse #3, Vero #1, BHK #2 | KP940141 | KT030661 |

ARC-OVI – Agricultural Research Council – Onderstepoort Veterinary Institute
ERC – Equine Research Centre, University of Pretoria.

### *Field Strains of AHSV*

In a previous study *(61)*, blood samples collected from pyrexic horses with signs typical of AHS *(60)* between 1 January 2011 and 31 May 2012 were subjected to an AHSV group specific RT-qPCR assay and VI. These samples were collected by veterinarians as part of routine diagnostic testing procedures, and ethical approval for the testing of these samples was obtained from the University of Pretoria's Animal Use and Care Committee (AUCC) according to the South African National Standard (SANS 10386: 2008) for the care and use of animals for scientific purposes. These samples consisted of 156 which were positive by both group specific AHSV RT-qPCR and VI, and a further 184 samples which were positive by AHSV RT-qPCR but negative by VI *(61)*. In the current study, all 156 samples that were positive by AHSV RT-qPCR and VI, as well as all samples that were VI negative but positive by AHSV RT-qPCR with a Cq < 33 (n = 135) were selected for analysis using AHSV TS RT-qPCR assays. An additional 7 blood samples collected from horses that were VI negative but confirmed to have AHSV infection by group specific RT-qPCR between 2009 and 2010 *(176)*, and stored at 4 °C for more than two years, were also tested using the AHSV TS RT-qPCR assays.

### *AHSV TS RT-qPCR Assay development*

Sequences encoding VP2 of each AHSV type available at the National Centre for Biotechnology Information's GenBank® website (www.ncbi.nlm.nih.gov) were analysed collectively and separately, and unique regions were identified as targets for primers and probes which were designed using Primer Express v3 (Lifetech). Oligonucleotides were



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synthesized by Lifetech. The sequences of the type specific primers and probes were evaluated *in silico* to ensure no cross-reactions with non-target AHSV types. Primer probe combinations for 3 AHSV types were combined into 3 triplex reactions. Triplex 1 included oligonucleotides for types 1, 3 and 4, which are the types included in bottle 1 of the Onderstepoort Biological Products AHS vaccine and which have been shown to be serologically distinct on neutralisation tests. Triplex 2 included oligonucleotides for types 2, 5 and 9 and Triplex 3 included oligonucleotides for types 6, 7 and 8. These triplexes were constituted to ensure that types which have been shown to cross-react on neutralisation tests were separated (types 1 and 2; 3 and 7; 5 and 9; 6 and 8). The sequences of the primers and probes and details of the triplex combinations are provided in Table 3-2.



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**Table 3-2: African horse sickness virus type specific primers and minor groove binding (MGB) probes for the three triplex assays.**

| Multiplex | Type | Primer/Probe | Sequence |
|---|---|---|---|
| 1 | 1 | Forward Primer | 5'-TGAACATAAACAAACGGTGAGTGA |
| | | Reverse Primer | 5'-GGTTAGAGGCGCTCGGTTCT |
| | | MGB Probe | 5'-FAM-CAGTTGAAAAAGAAACAAG |
| | 3 | Forward Primer | 5'-CAAATAATGGTACGTGGAGTAAGCA |
| | | Reverse Primer | 5'-TTCTTCTTTTGTTCCTCGTTCAAA |
| | | MGB Probe | 5'-VIC-AAAGCGGAAGTTAAGAAG |
| | 4 | Forward Primer | 5'-CATATAAAGGAGGTAACCGAGAAACTG |
| | | Reverse Primer | 5'-GGCATGGTTGTCCTCCATTT |
| | | MGB Probe | 5'-NED-AGAAAGCGCAAACCG |
| 2 | 2 | Forward Primer | 5'-ACATTGATAGGTTTAGCCGGACTT |
| | | Reverse Primer | 5'-CACTTTTTGTTTGTGTTCGTTCCA |
| | | MGB Probe | 5'-FAM-CAAGAYGAATATTGATCCAA |
| | 5 | Forward Primer | 5'-ACAAGAAAAAGGTACAAGAGCAGTTAGA |
| | | Reverse Primer | 5'-CCATTACTTTATACGGTTCGTTATTGTT |
| | | MGB Probe | 5'-VIC-AGGCGCAAAAGAA |
| | 9 | Forward Primer | 5'-CAGAGAGAGGATGCAGAAAGAACA |
| | | Reverse Primer | 5'-CGCCATCAACTTGGATCTTTAAG |
| | | MGB Probe | 5'-NED-AGCGCGAATTCCAA |
| 3 | 6 | Forward Primer | 5'-TTAATCCGAACCACCAAACG |
| | | Reverse Primer | 5'-GAGGTTTATTATTGTTGCCTTGC |
| | | MGB Probe | 5'-FAM-TGATCAAATGAATCGTGCGC |
| | 7 | Forward Primer | 5'-GATGGCGGAAAAGCTAAAGGA |
| | | Reverse Primer | 5'-GGCACTAGCATCGGACGATT |
| | | MGB Probe | 5'-VIC-AGCAACAGAAAAAC |
| | 8 | Forward | 5'-ACGGCGAAAATTGGAAAAAA |
| | | Reverse | 5'-TGCGCTTCATTCAAACGTTCT |
| | | MGB Probe | 5'-NED-ATAAGGCGGAAGTCC |

FAM , VIC, NED – Fluorescent dyes for the probes

### *RNA Extractions and RT-qPCR*

Nucleic acid extraction from equine blood samples and the AHSV reference viruses was performed using a Kingfisher 96 magnetic particle processor (Thermo Fisher Scientific Inc.) and the MagMAX™ Pathogen RNA/DNA kit (Applied Biosystems part number 4462359) according to the manufacturer's recommendations with slight modifications as described previously *(61)*. Group specific RT-qPCR assays were performed immediately after extraction and the plates containing the remaining eluates were stored at minus 20°C until the group specific RT-qPCR assays were completed. Stored nucleic acid extracts were then thawed and 5 µl of the eluate was transferred from the elution plate to each of 3 separate wells on a PCR plate and 5 µl of each of the 3 triplex primer probe mixes were added to



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each of these wells. The forward and reverse primer concentrations were limited to 200nM for each AHSV type and the probe concentration for each AHSV type was 120nM in the final PCR reaction volume. The plate was sealed with foil and heated at 95°C for 1 min (ABI GENEAMP PCR System 9700) to denature dsRNA in the eluate. The plate was then frozen at -20°C for 5 min. AHSV TS RT-qPCR assays were performed by adding 15 µl of VetMax™-Plus One-Step RT-PCR mastermix (Applied Biosystems part number 4415328) to each well on a 96-well PCR plate. The plate was sealed with a transparent plate sealer and the RT-qPCR was performed following the manufacturer's recommended conditions of 48°C for 10 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 45 sec on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Fluorescence was measured during the 60°C annealing/extension step and samples were classified as positive if the normalised fluorescence for the AHSV TS RT-qPCR assay exceeded a 0.1 threshold within 40 PCR cycles. The quantification cycle (Cq) was defined as the cycle number during which the fluorescence threshold was reached.

***Virus Isolation and Plaque Inhibition Test***

Virus isolation (VI) was performed essentially as previously described *(138)*. Briefly, blood was centrifuged at 2000-3500 rpm for 10min. The buffy coat was then harvested, diluted in PBS and dispensed into two sterile tubes and frozen at -80°C. This processed blood (0.2ml) was then inoculated onto a confluent monolayer of BHK-21 cells in 25cm$^2$ tissue culture flasks. Cell cultures were incubated at 37°C and observed daily for cytopathic effects (CPE). Cultures showing no CPE after 10–14 days were passaged by inoculating 0.2–0.5 ml of the culture onto a freshly prepared BHK-21 cell monolayer. When 70-100% of the cell monolayer showed CPE or had detached from the surface due to degeneration, the cells and supernatant were harvested and identified as AHSV by AHSV RT-qPCR *(61)*. Cultures showing no CPE after three passages were classified as negative for AHSV.

AHSV type determination was done using a plaque inhibition assay (PI) with fish spine beads containing individual type specific antisera, as previously described for Bluetongue virus *(81)* with slight modifications. Briefly, two sets of three confluent monolayers of Vero cells were prepared in 35 mm diameter cluster plates and inoculated with dilutions (1:50 and 1:100) of the virus sample. After adsorption for 1 h at 37°C in a 5% $CO_2$ gassed and humidified incubator, the inoculum was removed and replaced with 3 ml of an agarose overlay. Fish spine beads were immersed in type-specific antisera produced by inoculating individual sheep intravenously on two occasions at a 21 day interval with each of the 9 reference virus strains of AHSV supplied in 1995 by the OVI. The beads were placed



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in strict sequence on the surface of the overlay, after which the plates were returned to the incubator. Homologous neutralisation was clearly evident around the periphery of a bead by the absence of plaques after 5–6 days incubation.

## 3.4 Results

AHSV Type specific RT-qPCR assays developed for each of the 9 types of AHSV were evaluated using reference strains of each virus type (Table 3-3). This analysis confirmed that historic stocks of 3 of the 9 reference viruses were mixtures of two different AHSV types, specifically the stocks of AHSV-types 3, 7 and 8, also contained AHSV types 1, 3 and 5, respectively. In contrast, with the notable exception of the reference strain of AHSV type 3 that included a relatively low level of AHSV type 1, the newly propagated stocks of the reference viruses obtained in 2014 were monotypic.

**Table 3-3: Evaluation of two sets of reference strains of the 9 types of African horse sickness virus using group specific (AHSV RT-qPCR) and type specific (AHSV TS RT-qPCR) assays.**

| | Type | AHSV RT-qPCR | AHSV TS RT-qPCR | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Reference virus strains (1994) | 1 | 16.5 | 19.4 | * | * | * | * | * | * | * | * |
| | 2 | 16.8 | * | 26.4 | * | * | * | * | * | * | * |
| | 3 | 17.5 | 35.8 | * | 19.1 | * | * | * | * | * | * |
| | 4 | 16.9 | * | * | * | 20.8 | * | * | * | * | * |
| | 5 | 20.0 | * | * | * | * | 30.2 | * | * | * | * |
| | 6 | 19.6 | * | * | * | * | * | 25.2 | * | * | * |
| | 7 | 16.5 | * | * | 20.9 | * | * | * | 20.3 | * | * |
| | 8 | 17.1 | * | * | * | * | 20.7 | * | * | 21.6 | * |
| | 9 | 17.9 | * | * | * | * | * | * | * | * | 21.8 |
| Reference virus strains (2014) | 1 | 17.5 | 17.4 | * | * | * | * | * | * | * | * |
| | 2 | 18.8 | * | 21.5 | * | * | * | * | * | * | * |
| | 3 | 19.9 | 36.5 | * | 18.9 | * | * | * | * | * | * |
| | 4 | 19.4 | * | * | * | 21.5 | * | * | * | * | * |
| | 5 | 16.0 | * | * | * | * | 15.8 | * | * | * | * |
| | 6 | 20.2 | * | * | * | * | * | 21.6 | * | * | * |
| | 7 | 19.2 | * | * | * | * | * | * | 18.7 | * | * |
| | 8 | 18.8 | * | * | * | * | * | * | * | 18.9 | * |
| | 9 | 19.6 | * | * | * | * | * | * | * | * | 20.9 |



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Under the conditions described, samples can be extracted and evaluated by AHSV RT-qPCR within 4 hours after their arrival at the laboratory, as previously shown *(61)*. In this study the AHSV TS RT-qPCR was done on eluates of previously extracted samples in approximately 2 hours.

The 291 horse blood samples that were determined to be positive by AHSV RT-qPCR were also evaluated using the AHSV TS RT-qPCR assays (Table 3-4). Of these, 156 samples were VI positive with virus type determined by PI. All 9 types of AHSV were isolated from these 156 samples, and there was concordance between the types determined by PI with the results of the AHSV TS RT-qPCR assays. AHSV type was also determined by AHSV TS RT-qPCR for the 135 samples that were VI negative but AHSV RT-qPCR positive. Results of the group-specific AHSV RT-qPCR, each AHSV TS RT-qPCR and the PI for each of the 291 samples included in this study are provided as supplementary material (APPENDIX A)

**Table 3-4: African horse sickness virus types identified in equine blood samples by type specific AHSV TS RT-qPCR from cases of African horse sickness confirmed by group specific AHSV RT-qPCR.**

| Type | AHSV RT-qPCR positive and VI positive | AHSV RT-qPCR positive and VI negative | Combined |
|---|---|---|---|
| **1** | 22 | 23 | 45 |
| **2** | 9 | 26 | 35 |
| **3** | 5 | 1 | 6 |
| **4** | 11 | 12 | 23 |
| **5** | 11 | 13 | 24 |
| **6** | 20 | 7 | 27 |
| **7** | 16 | 20 | 36 |
| **8** | 58 | 31 | 89 |
| **9** | 4 | 2 | 6 |
| **Total** | **156** | **135** | **291** |

The geographic distribution of the viruses included in this study is provided in Figure 3-1 and Table 3-5. AHSV type 8 (AHSV8) was the most common type identified amongst field strains of AHSV tested in both 2011 and 2012, with some 30% of all positive samples containing this type. All 9 AHSV types were identified among samples from southern Africa evaluated in 2011, whereas neither AHSV1 nor AHSV9 was identified in 2012. AHSV type was also determined for 7 VI negative but AHSV RT-qPCR positive archived equine blood samples collected in a previous study *(176)*. Of the 7 samples tested, 3 samples tested positive for AHSV2, 2 samples tested positive for AHSV5, 1 for AHSV1 and 1 for AHSV4 (Table 3-6).



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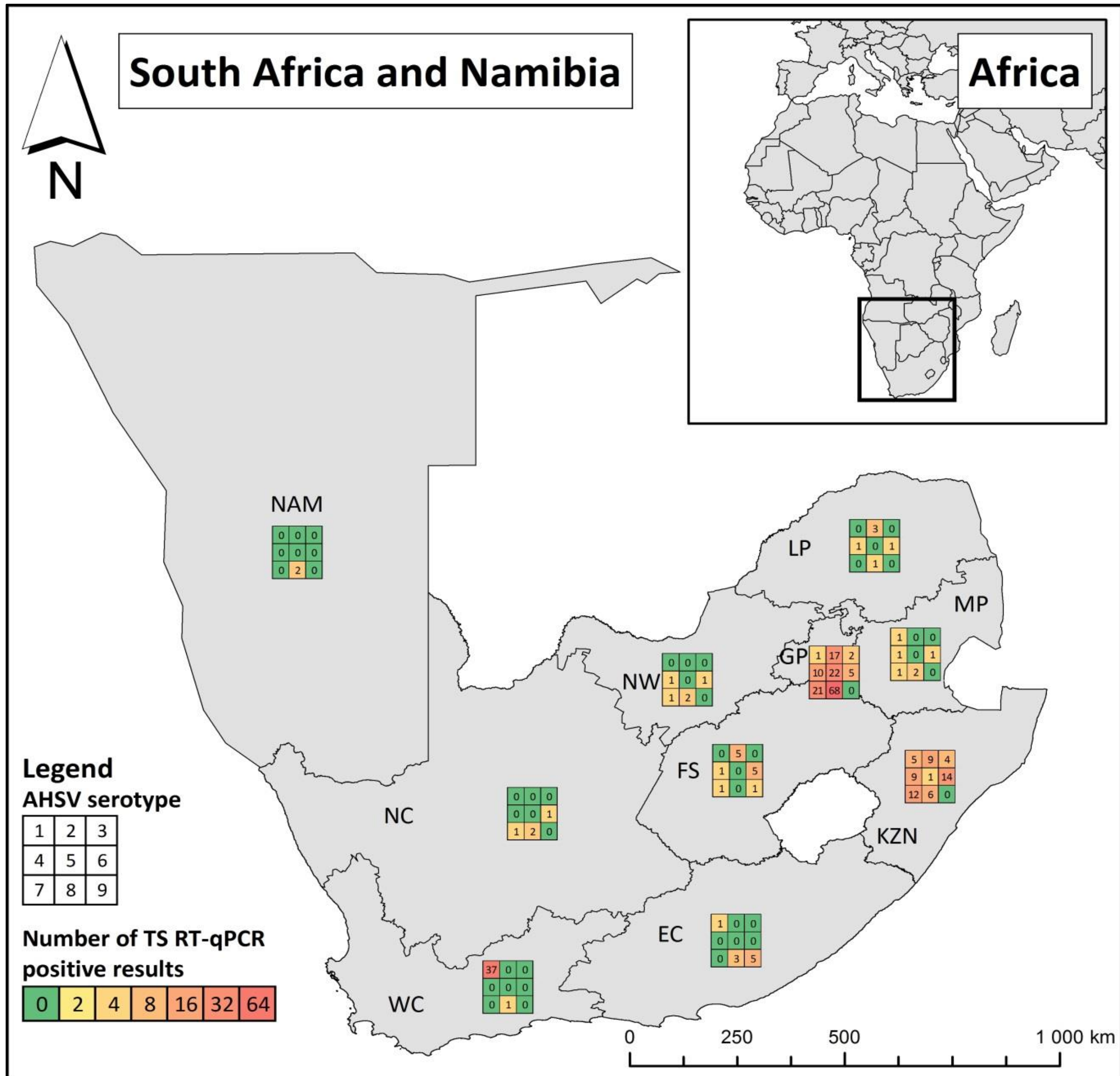


**Figure 3-1: A map of Namibia and South Africa with its provinces. The labels on the map refer to Namibia (NAM) and the nine provinces of South Africa, namely Eastern Cape (EC), Free State (FS), Gauteng (GP), KwaZulu-Natal (KZN), Limpopo (LP), Mpumalanga (MP), North West (NW),Northern Cape (NC) and the Western Cape Province (WC). The total number of positive AHSV TS RT-qPCR samples per type for samples collected between 1 January 2011 and 31 May 2012 is depicted for each area.**



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**Table 3-5: A summary of the total number of samples positive by TS specific RT-qPCR and virus isolation. The tables indicate ST RT-qPCR positive cases and viral isolation positive cases for each TS of African horse sickness in each province of South Africa and Namibia (see Figure 3-1) during 2011 and 2012.**

TS 1

| Province | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | Total positive | Total isolated | % isolated | Total positive | Total isolated | % isolated |
| EC | 1 | 0 | 0% | | | |
| FS | | | | | | |
| GP | 1 | 0 | 0% | | | |
| KZN | 5 | 1 | 20% | | | |
| LP | | | | | | |
| MP | 1 | 1 | 100% | | | |
| NAM | | | | | | |
| NW | | | | | | |
| NC | | | | | | |
| WC | 37 | 20 | 54% | | | |
| Total | 45 | 22 | 49% | 0 | 0 | 0% |

TS 6

| Province | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | Total positive | Total isolated | % isolated | Total positive | Total isolated | % isolated |
| EC | | | | | | |
| FS | 5 | 4 | 80% | | | |
| GP | 3 | 2 | 67% | 2 | 1 | 50% |
| KZN | 13 | 10 | 77% | 1 | 1 | 100% |
| LP | | | | 1 | 1 | 100% |
| MP | | | | 1 | 1 | 100% |
| NAM | | | | | | |
| NW | 1 | 0 | 0% | | | |
| NC | 1 | 0 | 0% | | | |
| WC | | | | | | |
| Total | 23 | 16 | 70% | 5 | 4 | 80% |

TS 2

| Province | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | Total positive | Total isolated | % isolated | Total positive | Total isolated | % isolated |
| EC | | | | | | |
| FS | 5 | 0 | 0% | | | |
| GP | 15 | 4 | 27% | 2 | 0 | 0% |
| KZN | 8 | 1 | 13% | 1 | 1 | 100% |
| LP | 1 | 0 | 0% | 2 | 2 | 100% |
| MP | | | | | | |
| NAM | | | | | | |
| NW | | | | | | |
| NC | | | | | | |
| WC | | | | | | |
| Total | 29 | 5 | 17% | 5 | 3 | 60% |

TS 7

| Province | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | Total positive | Total isolated | % isolated | Total positive | Total isolated | % isolated |
| EC | | | | | | |
| FS | 1 | 1 | 100% | | | |
| GP | 20 | 10 | 50% | 1 | 0 | 0% |
| KZN | 12 | 5 | 42% | | | |
| LP | | | | | | |
| MP | 1 | 0 | 0% | | | |
| NAM | | | | | | |
| NW | 1 | 0 | 0% | | | |
| NC | 1 | 0 | 0% | | | |
| WC | | | | | | |
| Total | 36 | 16 | 44% | 1 | 0 | 0% |

TS 3

| Province | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | Total positive | Total isolated | % isolated | Total positive | Total isolated | % isolated |
| EC | | | | | | |
| FS | | | | | | |
| GP | | | | 2 | 2 | 100% |
| KZN | 3 | 2 | 67% | 1 | 1 | 100% |
| LP | | | | | | |
| MP | | | | | | |
| NAM | | | | | | |
| NW | | | | | | |
| NC | | | | | | |
| WC | | | | | | |
| Total | 3 | 2 | 67% | 3 | 3 | 100% |

TS 8

| Province | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | Total positive | Total isolated | % isolated | Total positive | Total isolated | % isolated |
| EC | 3 | 1 | 33% | | | |
| FS | | | | | | |
| GP | 65 | 45 | 69% | 3 | 3 | 100% |
| KZN | 5 | 2 | 40% | 1 | 1 | 100% |
| LP | | | | 1 | 0 | 0% |
| MP | | | | 2 | 2 | 100% |
| NAM | | | | 2 | 1 | 50% |
| NW | 2 | 1 | 50% | | | |
| NC | 2 | 1 | 50% | | | |
| WC | 1 | 0 | 0% | | | |
| Total | 78 | 50 | 64% | 9 | 7 | 78% |

TS 4

| Province | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | Total positive | Total isolated | % isolated | Total positive | Total isolated | % isolated |
| EC | | | | | | |
| FS | 1 | 0 | 0% | | | |
| GP | 5 | 3 | 60% | 5 | 4 | 80% |
| KZN | 9 | 2 | 22% | | | |
| LP | | | | 1 | 1 | 100% |
| MP | | | | 1 | 1 | 100% |
| NAM | | | | | | |
| NW | 1 | 0 | 0% | | | |
| NC | | | | | | |
| WC | | | | | | |
| Total | 16 | 5 | 31% | 7 | 6 | 86% |

TS 9

| Province | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | Total positive | Total isolated | % isolated | Total positive | Total isolated | % isolated |
| EC | 5 | 4 | 80% | | | |
| FS | 1 | 0 | 0% | | | |
| GP | | | | | | |
| KZN | | | | | | |
| LP | | | | | | |
| MP | | | | | | |
| NAM | | | | | | |
| NW | | | | | | |
| NC | | | | | | |
| WC | | | | | | |
| Total | 6 | 4 | 67% | 0 | 0 | 0 |

TS 5

| Province | 2011 | | | 2012 | | |
|---|---|---|---|---|---|---|
| | Total positive | Total isolated | % isolated | Total positive | Total isolated | % isolated |
| EC | | | | | | |
| FS | | | | | | |
| GP | 21 | 10 | 48% | 1 | 0 | 0% |
| KZN | | | | 1 | 1 | 100% |
| LP | | | | | | |
| MP | | | | | | |
| NAM | | | | | | |
| NW | | | | | | |
| NC | | | | | | |
| WC | | | | | | |
| Total | 21 | 10 | 48% | 2 | 1 | 50% |



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**Table 3-6: Results of Group Specific (AHSV RT-qPCR) and type specific (AHSV TS RT-qPCR) assays performed on archived blood samples from clinical and subclinical cases of African horse sickness.**

| Sample | AHSV RT-qPCR | AHSV TS RT-qPCR | |
|---|---|---|---|
| | Cq | Type | Cq |
| A1001332 | 31.9 | 2 | 32.5 |
| A1001341 | 26.5 | 2 | 27.6 |
| A1001347 | 36.9 | 5 | 36.0 |
| A1001317 | 23.5 | 1 | 23.6 |
| A1001319 | 36.5 | 5 | 36.7 |
| A1001323 | 28.2 | 4 | 31.6 |
| A1001328 | 27.8 | 2 | 29.1 |

## 3.5 Discussion

We have developed AHSV TS RT-qPCR assays that were then characterized for applicability and practicability *(26)* using a large group of blood samples collected from horses that were naturally infected with AHSV. All 9 types of AHSV were represented in these samples, which include a wide variety of field strains of the virus that were circulating in southern Africa in 2011 and 2012. Importantly, these assays have been evaluated on blood samples rather than tissue culture propagated viruses. The multiplexing of the primers in groups of three allows for individual samples to be tested in three wells as opposed to nine individual wells, reducing the number of steps required as well as minimizing the reagents required for each sample tested.

Applications of AHSV TS RT-qPCR showed that 3/9 of the 1995 reference strains of AHSV were not monotypic, whereas only 1/9 of the 2014 references strains were not monotypic (type 3). The PI test used sera generated using the 1995 reference strains. Although cross reactivity occurs between types (particularly between 3 and 7, and 5 and 8) in serotyping assays using antisera generated with the 1995 reference viruses this does not preclude experienced virologists from interpreting these assays (G.H. Gerdes and P.G. Howell, Personal Communication). AHSV TS RT-qPCR assays applied to the 2014 reference strains indicate that type 3 includes type 1 at a low level. Whilst this observation could be due to lower specificity of the AHSV type 1 TS RT-qPCR resulting in a low positive signal against the AHSV type 3 reference strain this is not supported by the *in silico* analysis of primer and probe specificity and cross reactions were not observed in any of the type 3 field samples. Interestingly, the attenuated live virus AHS type 3 vaccine strain derived from this AHSV type 3 reference strain has recently been shown to be a reassortant between AHSV types 1 and 3 *(66)* suggesting that the prototype virus stock from which this vaccine strain was derived contained viruses of both type 1 and 3.



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Whilst there was concordance between the AHSV TS RT-qPCR and PI results in this study, the confirmation that 3 of the 1995 reference antigens were not monotypic meant that these data could not be used to reliably determine the diagnostic accuracy of the assays. Until such time as authenticated monotypic reference viruses for all 9 types are available and monotypic antisera have been generated such studies cannot be completed but further characterisation of these assays using appropriate methods *(26)* is warranted.

Concurrently with our development of AHSV TS RT-qPCR assays, Bachanek-Banowska et al *(10)* described nine individual TS RT-qPCR assays that were characterised using virus isolates from the Orbivirus Reference Collection at the Pirbright Institute. These included cell culture passaged isolates of reference strains of each of the nine AHSV types, field strains representing six of the nine AHSV types, and 4 vaccine strains. Relevant to all AHSV TS RT-qPCR assays, Manole et al. *(102)* recently described a laboratory-derived strain of AHSV-7 with an in-frame deletion of 225 amino acids in VP2 (AHSV7-tVP2). The deleted region of AHSV7-tVP2 includes one of the predicted major neutralisation determinants of AHSV, thus the AHSV-7 type-specific primers and probes included in both our AHSV TS RT-qPCR assay and that of Bachanek-Banowksa et al *(10)* target an area within this deleted region and would thus yield false negative results. Nevertheless, the group specific RT-qPCR assay results would be unaffected so serotyping of such deleted viruses would require alternative assays such as VI and PI.

The current tests prescribed by the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals for international trade are serologically based and a group specific RT-qPCR is given as an alternate assay *(183)*. The use of group-specific AHSV RT-qPCR in conjunction with AHSV TS RT-qPCR assays provides an attractive strategy for improved AHS surveillance and outbreak response protocols. Similarly, the use of these rapid molecular diagnostic assays will lead to a better understanding of the epidemiology of AHS, as the applicability and practicability of the AHSV TS RT-qPCR are superior to those of traditional VI and PI assays for virus serotyping. It has previously been shown that AHSV viral nucleic acid can persist for several months in the blood of horses that survive AHSV infection *(137, 176)*. Importantly, virus type was readily determined in samples that were positive by AHSV RT-qPCR but negative by VI. Use of both assays in tandem will be invaluable in expediting outbreak responses, such as those that have occurred in the AHS surveillance zone of the AHS Control Area of South Africa *(56)*.



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## 3.6 Conclusion

In summary, we describe three triplex AHSV TS RT-qPCR assays that can be applied directly to nucleic acid extracted from blood samples collected from AHSV infected horses. Specifically, the use of these assays in conjunction with a previously described group specific AHSV RT-qPCR assay with documented diagnostic accuracy can expedite investigation of AHS outbreaks and guide response strategies such as vaccination. Similarly, these assays may be useful for AHSV surveillance and epidemiological investigations.



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# CHAPTER 4

## Evaluation of the use of foals for active surveillance in an African horse sickness containment zone during the season following an outbreak.

### 4.1 Abstract

An outbreak of African horse sickness (AHS) occurred in the AHS surveillance zone of the Western Cape Province in the summer of 2011. The outbreak started in the town of Mamre in the Malmesbury Magisterial District and was effectively confined within a containment zone defined in this area. This outbreak resulted in the disruption of the export of horses directly from South Africa to AHS free countries. As part of their post-outbreak surveillance programme within the containment zone, the Directorate of Veterinary Services in the Western Cape Province collected serum and whole blood samples from identified (by microchip) foals born in the Mamre district after the end of the outbreak. These samples were evaluated by AHS virus (AHSV)-specific serological assay and the results were compared with those obtained using a real-time reverse transcription PCR (RT-qPCR) assay. The aim of this study was to show using both serological and molecular methods that AHSV was not transmitted to any of these previously unvaccinated (sentinel) foals in the year subsequent to the outbreak in the Mamre area.

### 4.2 Introduction

African horse sickness (AHS) is a notifiable disease in South Africa and is a life threatening disease of equids caused by African horse sickness virus (AHSV), a member of the genus *Orbivirus* in the family *Reoviridae*. The virus is transmitted to horses by midges (*Culicoides* spp.) and the disease is most prevalent during the time of year, and in areas where *Culicoides spp*. are most abundant, i.e. in late summer in the summer rainfall areas of the country *(30)*. In Southern Africa, the “high-risk” period for AHSV infection is late summer to early autumn, specifically, from February to the end of May.

The area around the Cape of Good Hope in South Africa has historically been free from AHS, with outbreaks occurring only intermittently since 1719. In 1997, South Africa declared AHS a controlled animal disease in terms of the Animal Diseases Act (Act No 35 of 1984) and defined part of the Western Cape Province as an AHS controlled area. This area comprises three different but contiguous zones (Figure 4-1), namely: 1) the AHS free zone encompassing metropolitan Cape Town, 2) the AHS surveillance zone, and 3) the AHS



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protection zone *(157)*. The rest of South Africa is regarded as AHS infected (AHSV endemic) and movement of equines into this controlled area is strictly regulated *(16)*.

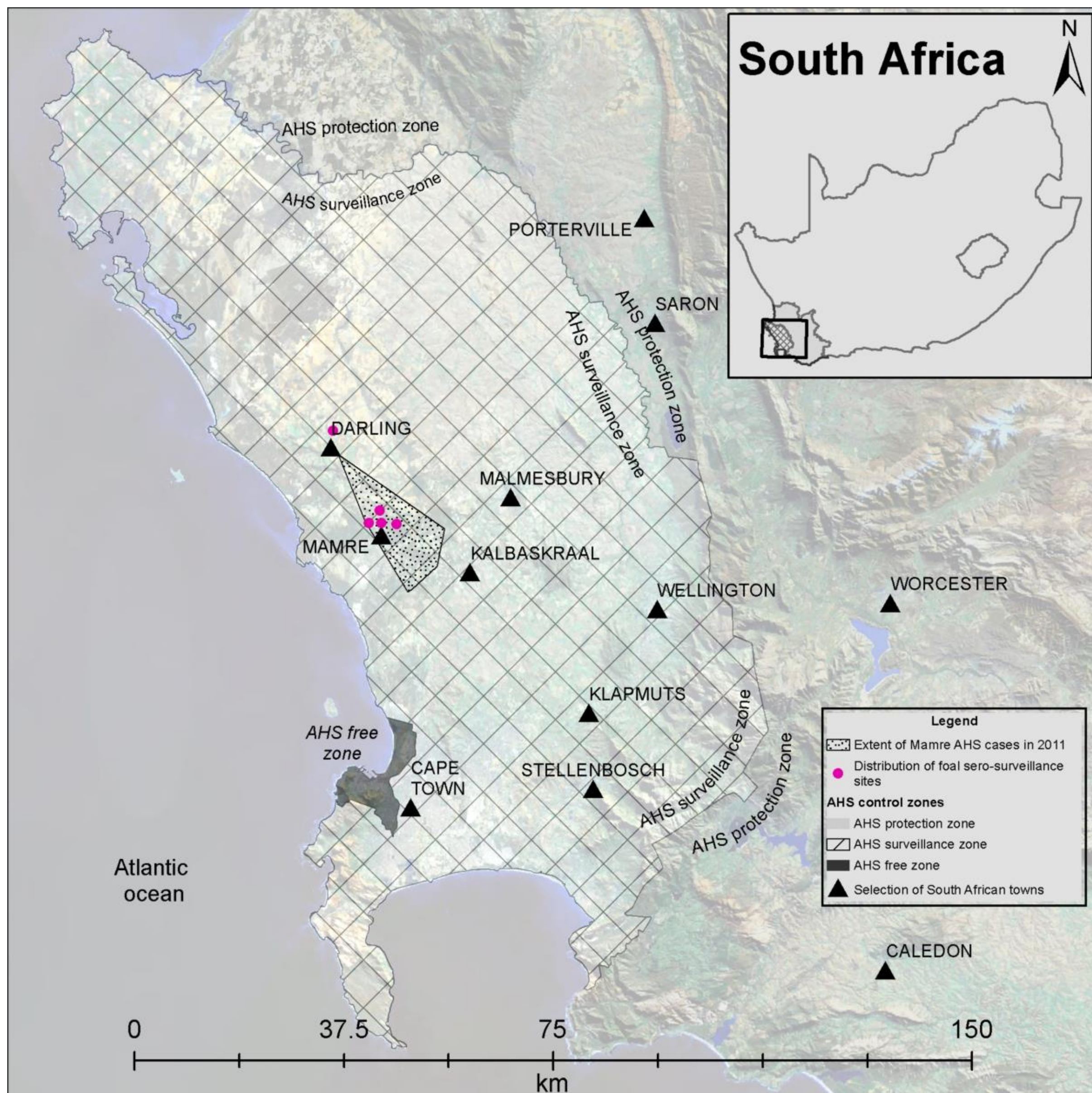


**Figure 4-1: A map showing the extent of the Mamre African horse sickness (AHS) outbreak in 2011, as well as the locations of the animals that were sampled in this study. The AHS free, surveillance and protections zones of the AHS controlled area are indicated.**

An outbreak of AHS occurred in the AHS surveillance zone of the AHS controlled area of the Western Cape Province in the summer of 2011. Illegal movement of an AHSV-infected animal into the surveillance zone was believed to have precipitated the outbreak at the time of investigation *(56)*. The initial confirmed case was identified on the 26th February 2011 and with the last confirmed case occurred on 23rd June 2011. Partial, direct sequencing of the S10 and L2 genes of the circulating virus confirmed the outbreak virus to be AHSV type 1 *(56)*. The 2011 outbreak started in the town of Mamre in the Malmesbury Magisterial District,



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and was effectively confined to a containment zone within this area by movement control of all equids and a mass vaccination campaign. Of the 73 confirmed cases of AHS, as reported to the OIE, that occurred during this outbreak, 4 subclinical cases were confirmed in horses showing no obvious clinical symptoms of AHS but that tested positive for AHSV by both RT-qPCR and virus isolation assays. Subclinical AHSV infection of horses has also been described previously in horses' resident in the AHS infected zone of South Africa *(176)*. This RT-qPCR assay has also been shown to be effective as a surveillance tool to detect horses with AHSV infections whether they are showing clinical symptoms or not *(56, 176)*.

The World Organisation for Animal Health's (OIE) Terrestrial Animal Health Code recommends that surveillance for AHS identify either the occurrence of clinical signs related to AHS or evidence of infection with AHSV with or without clinical signs. The surveillance measures prescribed by the European Commission Decision (2008/698/EC) within the AHS free and surveillance zones include serological testing of sentinel animals, investigation of animals showing clinical signs of AHS, and documented necropsy and laboratory evaluation of any equine mortality event suspected of being due to infectious disease that occurs in these areas *(16)*. The use of serology as the sole testing method following an AHS outbreak in a containment zone is problematic as horses that were vaccinated or exposed to field virus during the outbreak test positive and so are not useful as sentinels. Similarly, serology has been shown to be unreliable as a surveillance tool in vaccinated horses resident in an AHS endemic area of South Africa *(175)*. The confirmation of subclinical cases of AHSV infection during this outbreak also highlights the need to develop surveillance systems to reliably identify subclinical cases of AHS during and subsequent to AHS outbreaks.

The aim of this study was to apply molecular and serological assays to samples collected from a group of foals born between September 2011 (more than 60 days after the last case reported in the outbreak) and January 2012 resident within the AHS containment zone of the 2011 Mamre AHS outbreak in order to show that AHSV was not transmitted to these foals in the potential AHSV transmission season subsequent to the outbreaks. This strategy provided a means of evaluating the effectiveness of the control programme instituted by the Directorate of Veterinary Services, Western Cape, as an emergency response to the outbreak. Furthermore the study provided an opportunity to compare serological and molecular surveillance techniques for AHS in horses following an outbreak, and the utility of foals as unvaccinated sentinels for AHS surveillance.



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## 4.3 Materials and Methods

### *Study Population*

Following the AHS outbreak in the Mamre area in 2011 the State Veterinarian at Malmesbury instituted a horse identification programme within the area, which consisted of the implantation of microchips and the completion of an equine identification form including both pictorial and written descriptions of individual animals. 322 horses belonging to 49 owners were identified in the Mamre area by the end of 2011. At the time of identification, owners of mares that appeared pregnant were asked to enrol any foals that were born from January through June 2012 as unvaccinated sentinels. The foals were identified by microchip and description, and serum and EDTA blood samples were collected from each foal monthly. Owners were incentivized to participate in the program, which resulted in the enrolment of 15 foals. The locations and details of the sentinel foals are provided in Figure 4-2 and Table 4-1, respectively. As part of the emergency response to the outbreak of AHS in Mamre in 2011, all equids within the immediate outbreak area were vaccinated against AHS using bottle 1 of the polyvalent AHSV live attenuated vaccine produced by Onderstepoort Biological Products, after confirmation that AHSV type 1 was responsible for the outbreak. Foals born after the conclusion of the outbreak (end of June 2011) were not vaccinated against AHS and, because the outbreak already was over by then, they also should not have been exposed to circulating field virus.



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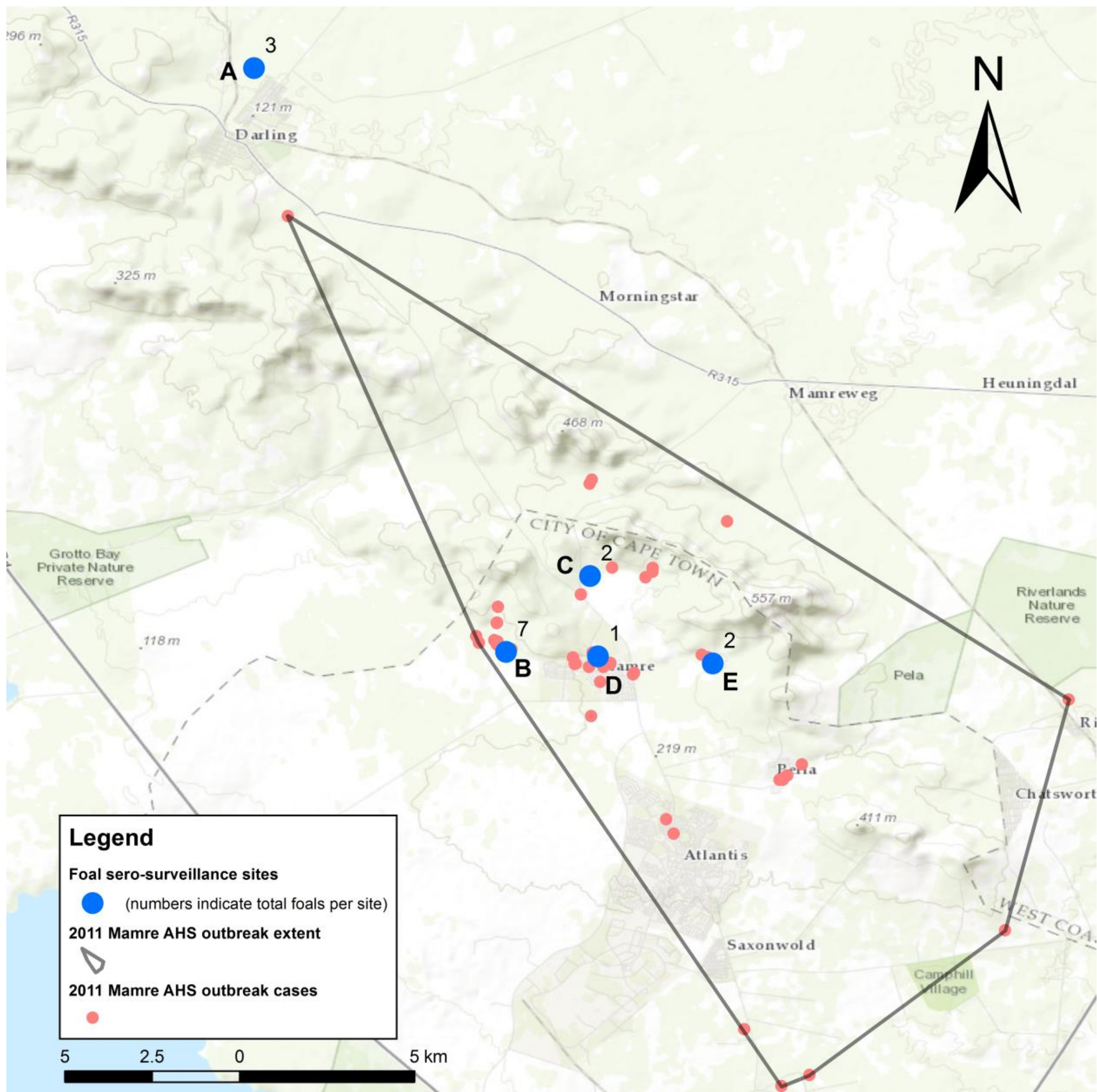


**Figure 4-2: A map showing the spatial distribution of the Holdings where the foal sentinels where situated (Blue dots) in relation to the confirmed cases of AHS (red dots) and the extent of the outbreak (polygon) during the 2011 Mamre AHS outbreak. Location A) Darling, B) Quarry, C) Skurwekraal, D) Mamre Town and E) Nature Reserve.**



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| Location | Horse Name | MOB | Microchip no |
|---|---|---|---|
| **A) Darling** | Bin Laden | Nov-11 | 710098100116035 |
| | London | Nov-11 | 710098100116289 |
| | Joy | Oct-11 | 710098100117868 |
| **B) Quarry** | Black Pearl | Oct-11 | 710098100118305 |
| | Lucy Foal | Oct-11 | 710098100116056 |
| | BelBel Foal | Dec-11 | 710098100117985 |
| | Vlam Foal | Dec-11 | 710098100116796 |
| | Optel Foal | Nov-11 | 710098100118082 |
| | Twister | Sep-11 | 710098100115772 |
| | Major | Dec-11 | 710098100118133 |
| **C) Skurwekraal** | Suzi | Dec-11 | 710098100119247 |
| | Kim | Dec-11 | 710098100116403 |
| **D) Mamre Town** | Jasmine Foal | Oct-11 | 710098100117922 |
| **E) Nature Reserve** | Sandy | Dec-11 | 710098100119098 |
| | Chelsea | Dec-11 | 710098100120184 |

**Table 4-1: A table showing the location name, identification (name and microchip number) and month of birth (MOB) of the foals used in this study.**

### *Sample collection and processing*

A whole blood sample in EDTA and a serum sample were collected in vacutainer tubes from each foal at the beginning of each month from January until June 2012. This period was chosen as it is extends from approximately a month prior to and a month after the estimated “high-risk” season for AHSV transmission. The sampling strategy and use of the foals for this project was granted ethical approval by the Animal Use and Care Committee of the University of Pretoria (Appendix G). The EDTA samples were processed and tested with an AHSV group specific RT-qPCR as previously described *(61)*. A Cq value > 37 was considered negative. Serum samples were subjected to an indirect ELISA (iELISA) to detect group specific antibodies to AHSV as previously described *(105)*. A percentage of the positive-control optical density (PP) value > 10 was considered positive.



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## 4.4 Results

In the Mamre outbreak of 2011, 11 of the 73 cases were under 1 year of age (personal communication: Western Cape Department of Agriculture). The total population for the Mamre outbreak area as depicted in Figure 4-2 was estimated at 447 equines at the time of the outbreak. Young horses therefore made up approximately 2.4% of the incidence of the outbreak (total incidence estimated at 16.3%) *(56)*. The estimated census for the same area at the time of this study was 650 equines; therefore 15 foals would represent 2.3% of the horse population. The distribution of the foals in relation to the outbreak extent was good, although coverage of the south-eastern area was lacking.

The RT-qPCR and iELISA results for each of the foals are summarised in Table 4-2.

**Table 4-2: Summary of results of the RT-qPCR assays performed on EDTA blood samples from each foal each month and of iELISA assays performed on serum samples from each foal at the beginning and end of the study. For the RT-qPCR, a Cq > 37 was considered negative. For the iELISA, a percentage positive value > 10 was considered positive. NS indicates that no samples were collected that month. The age in months (m) of each foal is given as the age at start of study in January 2012.**

| Name | Age (m) | Group Specific RT-qPCR | | | | | | iELISA | |
|---|---|---|---|---|---|---|---|---|---|
| | | Jan | Feb | March | April | May | June | Pre | Post |
| Bin Laden | 2 | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| London | 2 | Neg | Neg | Neg | Neg | Neg | Neg | 97 | Neg |
| Joy | 3 | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| Black Pearl | 3 | Neg | Neg | Neg | Neg | Neg | Neg | 27 | Neg |
| Lucy's Foal | 3 | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| BelBel's Foal | 1 | Neg | Neg | Neg | Neg | Neg | Neg | 21 | Neg |
| Vlam's Foal | 1 | Neg | Neg | Neg | Neg | Neg | Neg | 28 | Neg |
| Optel's Foal | 2 | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| Twister's Foal | 4 | Neg | Neg | Neg | Neg | Neg | Neg | 21 | Neg |
| Major | 1 | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| Suzi | 1 | Neg | Neg | Neg | Neg | Neg | Neg | 58 | Neg |
| Kim | 1 | Neg | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| Jasmine's Foal | 3 | NS | Neg | Neg | Neg | Neg | Neg | Neg | Neg |
| Sandy | 1 | NS | Neg | Neg | Neg | Neg | NS | 87 | 17 |
| Chelsea | 1 | NS | Neg | Neg | Neg | Neg | NS | 42 | 15 |

None of the foals tested positive on the group-specific RT-qPCR at any point during the study. No foals sero-converted during the study period although 8 of 15 (53%) were positive to AHSV by iELISA at the start of the study. Two of these 15 foals were still positive by iELISA at the conclusion of the study.



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## 4.5 Discussion

This study highlights the challenges to serological surveillance in any area where either vaccination or natural AHSV infection occurs. All the foals used in this study were born to dams that had been vaccinated during the outbreak response. The positive iELISA results from 8 foals likely reflects the presence of maternal antibody as all the foals were less than 4 months of age at the start of the study. By the end of the study the foal ages ranged from 6 - 9 months of age. The two iELISA positive foals were 6 months of age by this stage. Similarly, Crafford et al *(33)* previously reported that maternal antibody to AHSV persisted on average for 96 days (~3months) with a range from 62 days (~2months) to 128 days (4 months). Crafford et al also showed that a high percentage of Thoroughbred foals born to vaccinated dams were seropositive by iELISA for up to 4 months of age *(33)*. Collectively, these data support the use of older foals for surveillance as the use of foals older than 6 months avoids potential interference by maternal antibody. However, use of older (> 6 months of age) foals is complicated by the fact that this is the age when first vaccination is normally done in AHSV-endemic areas.

Agent identification testing using group specific RT-qPCR is useful for the detection of both clinical and subclinical cases of AHS. It has been shown previously that RNA can be detected in the blood of naturally AHSV-infected horses for prolonged periods, with subclinically infected animals still being RNA positive at least a month after infection *(176)*. Agent identification based surveillance is further complicated by the fact that RT-qPCR assays detect AHSV RNA in the blood of horses that are immunized with AHSV-LAVs for up to 12 weeks, especially in that of previously unvaccinated animals such as foals (Chapter 5). In contrast, the unvaccinated foals evaluated in the current study all remained negative by RT-qPCR assay for the duration of the study. Logically, only unvaccinated animals should be used as sentinels in agent identification based surveillance.

Sentinel animals are an integral part of targeted surveillance for AHS described in the OIE Animal Health Code. Sentinel animals should be previously unexposed equids that have not been vaccinated against AHSV, are managed at fixed locations, and observed and sampled regularly. Identification of seronegative adult horses for use as sentinels is difficult in areas where there has been vaccination or previous field outbreaks of AHS. In contrast, foals born after an outbreak are ideal sentinels for use in historically AHSV-free areas. The iELISA, the only serological technique currently available for group specific AHSV antibody detection in South Africa, is not quantitative *(105)*. This test provides a percentage positive value, which ultimately yields only a dichotomous (positive/ negative) result that is not quantitatively



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comparable between samplings of AHSV seropositive equids. This test is, therefore, only suitable for surveillance of seronegative horses.

In an AHS free zone or country, the OIE makes provision for a containment zone to be established in the event of limited outbreaks, in order that the impact of the outbreak on the entire country or zone is limited *(180)*. After the establishment of such a zone, increased passive and targeted surveillance must be implemented in the remaining free zone or country, as well as within the containment zone. If vaccination has occurred within the containment zone in response to the outbreak, identifying animals suitable for sentinel surveillance will be difficult to impossible. Foals born after an outbreak would be the most logical candidates. For this reason part of an outbreak response, and in fact part of the maintenance of a free zone or country would be to accurate know the location and census of potential breeding establishments, in order that these populations could be used in an outbreak response strategy.

In summary, results from this limited study following the 2011 outbreak of AHS in the Mamre area confirm that a sentinel foal surveillance system is suitable for the monitoring of the AHSV infection and transmission status of an area after an outbreak, or to demonstrate freedom from AHSV infection. This system should include monthly blood sample collections from each foal, which are then subjected to monthly serological (iELISA) and/or agent detection (RT-qPCR) testing. The use of unvaccinated foals as sentinels for virological surveillance requires that they be born during a time when there is no virus circulation. Furthermore, serological surveillance is potentially complicated by the presence of maternal antibody to AHSV in young foals, requiring the use of foals > 6 months of age. A surveillance strategy based on use of foals as sentinel animals could be considered following future AHS outbreaks in free areas or countries in order to provide data to support the subsequent reinstatement of an AHS free status.



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# CHAPTER 5

# Dynamics of African horse sickness virus nucleic acid and antibody in horses following immunisation with a commercial polyvalent attenuated live virus vaccine

## 5.1 Abstract

African horse sickness (AHS) is a frequently fatal disease of equids that is relevant to the global equine industry. Detection of AHS virus (AHSV) during outbreaks has become more rapid and efficient with the advent of group specific reverse transcriptase quantitative polymerase chain reaction (GS RT-qPCR) assays to detect AHSV nucleic acid. Use of GS RT-qPCR together with recently described type specific (TS RT-qPCR) assays can not only expedite diagnosis of AHS but also facilitate further evaluation of the dynamics of AHSV infection in the equine host. A potential limitation to the application of these assays is that they detect viral nucleic acid originating from any AHS live attenuated vaccine (LAV), which is the vaccine type routinely administered to horses in South Africa. The aim of this study was to contrast the dynamics and duration of the RNAaemia to the serological responses of horses following immunization with a commercial polyvalent AHSV-LAV using GS and TS RT-qPCR assays and serum neutralisation tests. The results of the study showed extended RNAemia in vaccinated horses, and that more horses tested positive on GS RT-qPCR with lower Cq values after receiving the AHSV-LAV containing types 1, 3 and 4 prior to the vaccine containing types 2, 6, 7 and 8, rather than when the vaccine combinations were reversed. Furthermore, lower Cq values were obtained when vaccines were administered 4 weeks apart as compared with a longer interval or 12 weeks apart. These findings are of particular relevance in regions where AHSV-LAVs are used as the use of these vaccines may complicate the accurate interpretation of diagnostic testing results.

## 5.2 Introduction

African horse sickness (AHS) is an infectious, non-contagious, arthropod-borne disease of equids caused by African horse sickness virus (AHSV) (genus Orbivirus, family Reoviridae). The AHSV genome includes 10 segments of double-stranded RNA *(23, 30, 114)*. AHSV infection in horses often results in mortality rates of up to 90 %. *(60)*. Foals born to immune mares acquire passive immunity by ingestion of colostrum, and maternally derived antibody progressively declines to undetectable levels at four to six months of age *(33)*. AHS is



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widespread in sub-Saharan Africa with all 9 types of AHSV occurring regularly in southern and eastern Africa.

Annual immunization with polyvalent live attenuated vaccine (LAV) is currently the mainstay for control of AHS in endemic areas of Africa. In South Africa, a polyvalent AHSV-LAV[1] is supplied as two separate vials: OBP comb1 and OBP comb2, which are administered at least three weeks apart. OBP comb1 includes AHSV types 1, 3 and 4 (AHSV-1, 3, and 4) and OBP comb2 includes AHSV-2, 6, 7 and 8. The whole genome sequences of the strains included in these vaccines are published *(66, 67)*. The current formulation, which does not include either AHSV-5 or 9, was introduced into use in 1994 *(171)*. Serological cross-reaction reportedly occurs between certain types: AHSV-1 with AHSV-2, AHSV-3 with AHSV-7, AHSV-6 with AHSV-9, AHSV-8 with AHSV-5, whereas AHSV-4 does not exhibit cross-reaction with other types. The different types are allocated to the two combinations based on these cross reactions *(42, 171)*. The immune response of horses to immunization with AHSV-LAVs has been investigated *(32, 134, 171)*, however the viral kinetics following immunization have not been characterized.

The objectives of this study were to determine the occurrence and duration of RNAaemia detected by GS RT-qPCR following immunization with a commercial polyvalent AHSV-LAV of: 1) weanlings following primary immunization, 2) yearlings following secondary immunization, and 3) adult mares with a history of multiple previous immunizations. Furthermore, the dynamics of the various AHSV-LAV types following immunization were characterized using type-specific RT-qPCR (TS RT-qPCR ) assays, which were also compared to type-specific serological responses as determined using serum neutralisation tests (SNT's).

## 5.3 Materials and Methods

### *Study Population*

Thoroughbred weanlings (*n=44)*, 7 to 10 months old at the start of the study, and brood mares (*n=22)* were used in this study. The horses were resident on a commercial stud farm

[1] manufactured by Onderstepoort Biological Products (OBP) Ltd



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within the AHS Controlled Area (CA) of the Western Cape Province of South Africa. No cases of AHS had been detected within a radius of at least 30km of this farm since the inception of the AHS CA in 1997 up to and including the period of this study. The weanlings were subjected to AHSV group-specific indirect ELISA (iELISA) tests *(105)* in January and June 2012, prior to the start of the study. Ethical approval for the sampling strategy and use of these animals was granted by the Animal Use and Care Committee of the University of Pretoria (Appendix E).

***Study Design***

The weanling foals in this study were randomly assigned to four groups (*n=11* weanlings per group*),* and received primary immunizations (with either OBP comb1 or OBP comb2) at either 4 or 12 week intervals with the AHSV-LAV according to the schedule detailed in Figure 5-1. The weanlings in groups I and II were also sampled as yearlings after their secondary immunizations, which occurred 16 weeks after initial immunisation.

Samples were collected from brood mares (*n=22)* that had previously received multiple AHS immunizations. The mares were immunized with OBP comb1 initially and OBP comb2 4 weeks later.

Whole blood (EDTA) samples were collected on the day of primary immunization (week 0) from all weanlings, and the brood mares, and then weekly as detailed in Figure 5-1. Serum samples were collected from the weanlings on the day of primary immunization (week 0) and weeks 4, 8, 12 and 16 after initial immunization.



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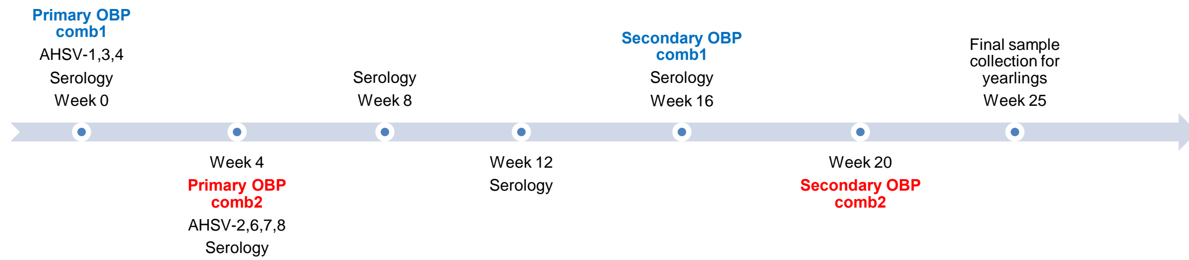
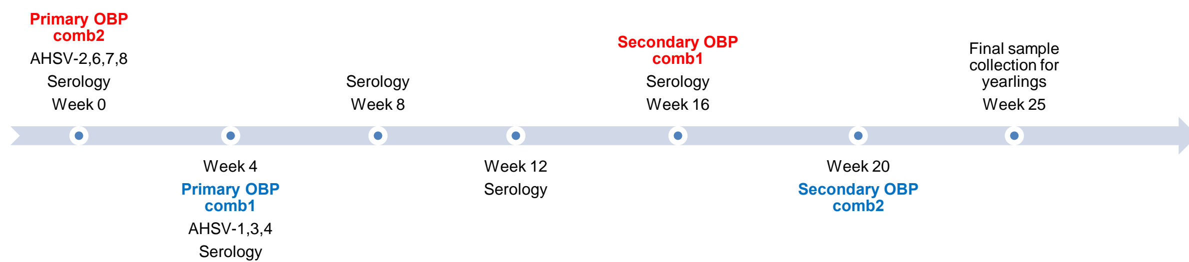
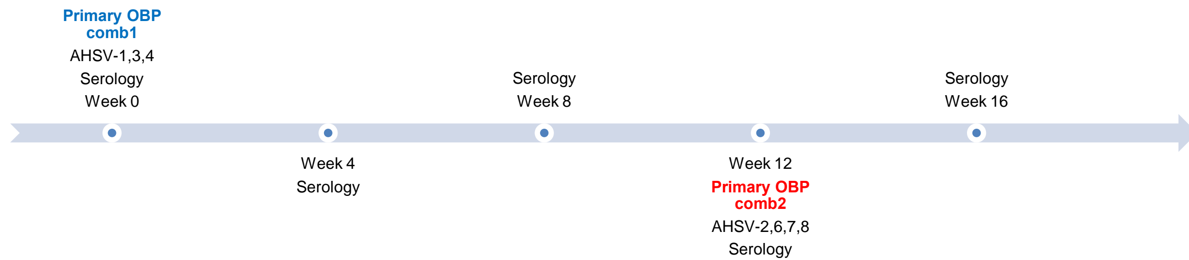
***Treatment Group I:***




***Treatment Group II:***




***Treatment Group III:***




***Treatment Group IV:***

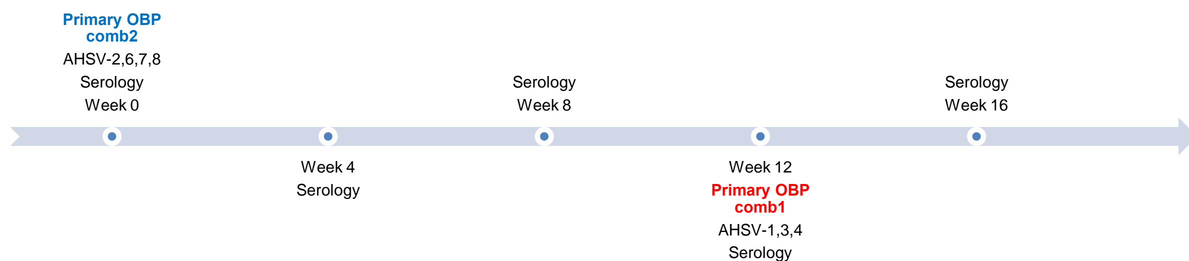



**Figure 5-1: Group Timelines depicting the vaccination schedule and serological sample collection times for each of the 4 weanlings treatment groups.**



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### ***Nucleic acid Detection***

*Group specific PCR*

A GS RT-qPCR assay with defined diagnostic sensitivity and specificity was used to quantify AHSV RNAaemia of whole blood samples as previously described *(61)*. Samples were classified as AHSV positive if the normalised fluorescence for the AHSV assay exceeded a 0.1 threshold within 37 PCR cycles *(61)*.

*Type specific PCR*

TS RT-qPCR assays were applied to samples that tested positive by the GS RT-qPCR with a Cq value < 33, as previously described *(177)*. Samples were classified as positive for a specific AHSV type if the normalised fluorescence for the specific TS RT-qPCR exceeded a 0.1 threshold within 40 PCR cycles.

The gene encoding VP2 of the AHSV-7 vaccine strain (AHSV-7Vacc) is truncated *(67)* and the primers and probe of the AHSV-7 TS RT-qPCR assay targets the deleted region *(177)*. Therefore, primers and a probe (Table 5-1) were designed to detect the truncated VP2 gene of the AHSV-7Vacc strain, as well as that of other field and laboratory strains of AHSV-7 available on Genbank. The sequences of the AHSV-7Vacc primers and probe were evaluated *in silico* to ensure there were no cross-reactions with other AHSV types.

**Table 5-1: African horse sickness virus (AHSV) type specific primers and minor groove binding (MGB) probe for AHSV type 7 including the vaccine strain with the truncated gene for VP2.**

| Type | Primer/Probe | Sequence |
|---|---|---|
| AHSV-7Vacc | Forward Primer | 5'-GCG AAA TAG AAC ACG TRA AGA CGA T |
| | Reverse Primer | 5'-ACA TAA TGA GGG AAC ACC GGA TA |
| | MGB Probe | 5'-VIC-TGA ACA AAT TAA ATG TGA GGG TG |

*AHSV-LAV virus detection*

OBP comb1 and OBP comb2 bottles were processed by extraction and GS RT-qPCR followed by TS RT-qPCR in the same manner as the EDTA blood samples.



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#### *Serum Neutralisation Tests*

Type specific antibodies were detected using SNT assays as previously described *(32, 33)*. Antibody titres are recorded as the reciprocal of the highest final dilution of serum that provided at least 50% protection of the Vero cell monolayer. A titre > 10 indicated positive results for that AHSV type. A four-fold increase in paired sample titres or a change from seronegative to seropositive indicated seroconversion *(63)*.

#### *Statistical Analysis*

The GS RT-qPCR median Cq value distributions for the different weanling groups over time were compared using the Wilcoxon rank-sum test. A *p-value* <0.05 was considered significant. The number of foals that seroconverted to each of the 9 types on SNT was compared between the different weanling groups using a two-way ANOVA test in R[2]. A *p-value* <0.05 was considered significant.

## 5.4 Results

#### *Weanling foals*

*iELISA Results*

In January 32/44 (73%) of the weanlings were seropositive by iELISA. The ages of the positive weanlings ranged from 56 - 149 days. All weanlings were seronegative by June.

*GS RT-qPCR results*

The kinetics of RNAemia as detected by GS RT-qPCR in immunized weanlings are provided in Figures 5-2, 5-3 and 5-4 and APPENDIX B (Tables B1 – B4). Figure 5-2 summarises the number of weanlings positive on GS RT-qPCR per week for each of the 4 groups. Figure 5-3 depicts the Cq value distributions of the GS RT-qPCR positive weanlings in each group.

[2] R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.



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Group I had a minimum median Cq value of 29.3 at week 3 after vaccination with OBP comb1. This was lower than the minimum median Cq values of the other groups. In group I there was also an individual weanling that was positive on GS RT-qPCR with a Cq value of 22.3 at week 4 (the same day that the weanling was immunised with OBP comb2). This was also lower than the minimum Cq value of any of the other weanlings in any of the other weanling groups.

Four (of 11) weanlings in group I were RT-qPCR positive at week 16 when they received their secondary yearling immunization with OBP comb1. Three (27%) of the weanlings in group I remained negative by GS RT-qPCR throughout the study. In group II, two weanlings were GS RT-qPCR positive at week 16 when they received their secondary yearling immunization with OBP comb1. Three weanlings (27%) in group II remained negative by GS RT-qPCR throughout. In group III all weanlings were negative on GS RT-qPCR by week 12 when immunization with OBP comb1 occurred. Only one weanling tested positive within the four week period following administration of OBP comb2. One weanling (9%) in group III did not test positive on GS RT-qPCR throughout. Three weanlings (27%) were positive at the time of secondary immunisation in group IV. Six (45%) tested positive with Cq values ≤ 37, but > 30 within the four weeks after yearling immunization with OBP comb1.



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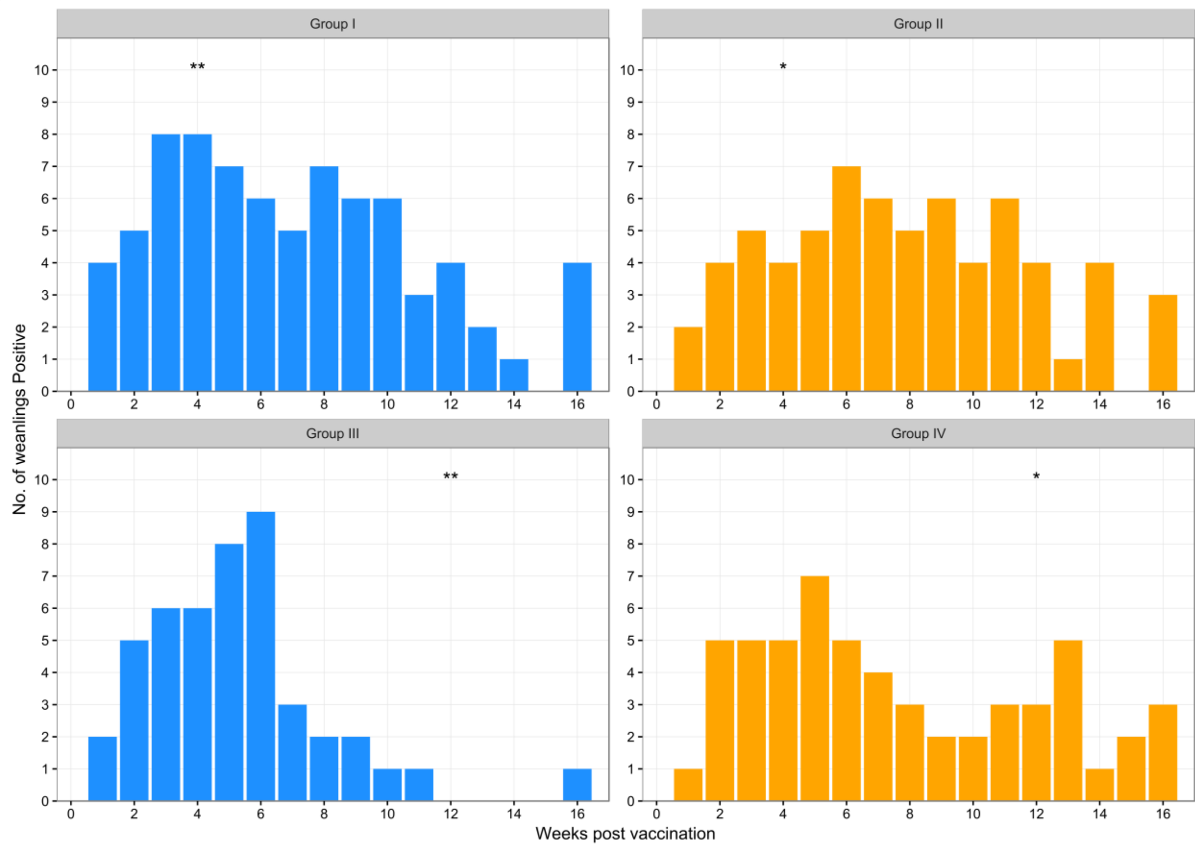


**Figure 5-2: Barplots showing the total number of weanlings positive on group specific RT-qPCR over time in each of the 4 weanling groups. Weanling group I initially received African horse sickness live attenuated vaccine combination 1 (OBP comb1) at week 0, and OBP comb2 4 weeks later (OBP comb2 is indicated by **). Weanling group II received OBP comb2 at week 0 and OBP comb1 4 weeks later (OBP comb1 is indicated by *). Weanling group III received OBP comb1 at week 0 and OBP comb2 12 weeks later (**). Weanling group IV received OBP comb2 at week 0 and OBP comb1 12 weeks later (*). The weanling groups that received OBP comb1 initially are indicated by blue bars. Weanling groups that received OBP comb2 initially are indicated by orange bars.**



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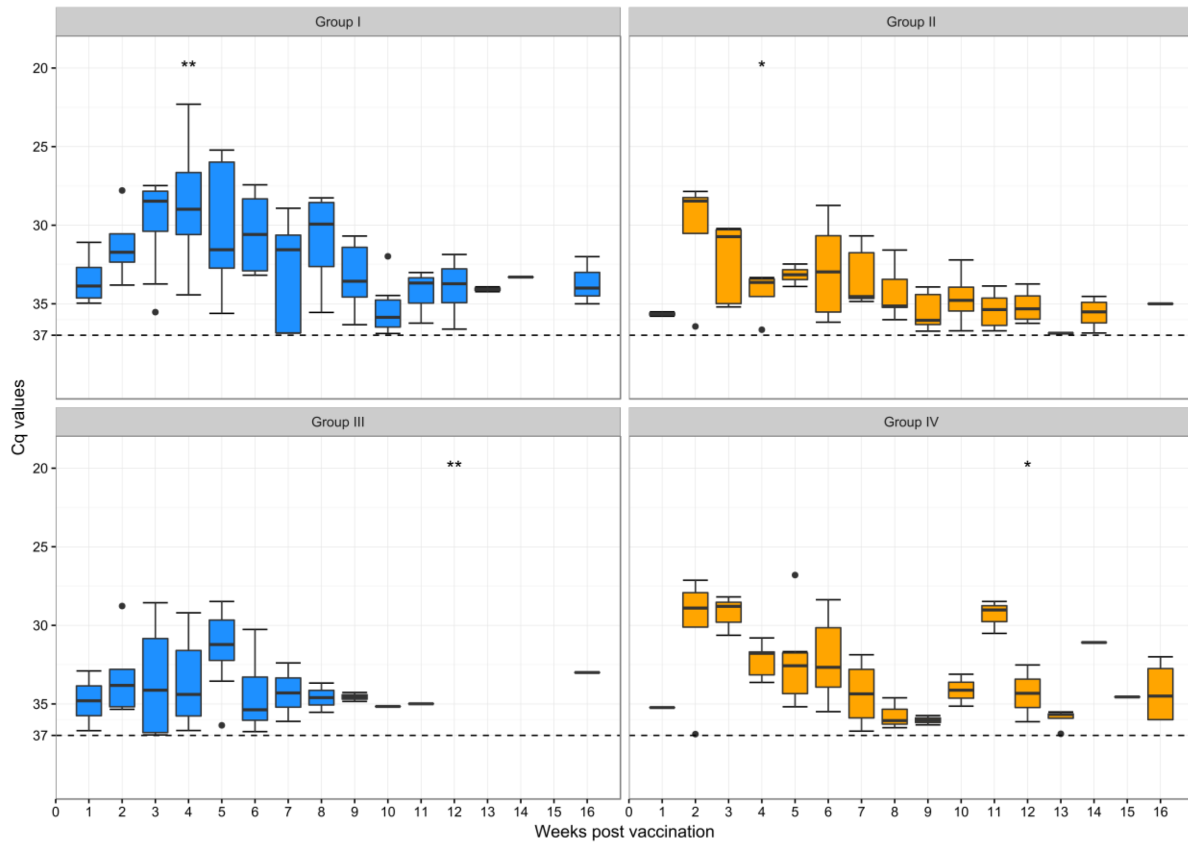


**Figure 5-3: A box and whisker plot describing the Cq value distributions of the GS RT-qPCR positive weanlings in each of the 4 AHS weanling groups (Groups I to IV). The solid line within the boxes represents the median Cq value. The outlier results are represented by the dots. Weanling group I received African horse sickness live attenuated vaccine combination 1 (OBP comb1) at week 0, and OBP comb2 4 weeks later (OBP comb2 is indicated by **). Weanling group II received OBP comb2 at week 0 and OBP comb1 4 weeks later (OBP comb1 is indicated by *). Weanling group III received OBP comb1 at week 0 and OBP comb2 12 weeks later (**). Weanling group IV received OBP comb2 at week 0 and OBP comb1 12 weeks later (*). The weanling groups that received OBP comb1 initially are indicated by blue bars. Weanling groups that received OBP comb2 initially are indicated by orange bars.**



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*TS RT-qPCR results*

Figure 5-4 summarises the number of weanlings that tested positive by TS RT-qPCR for each AHSV type, and the time period the type was detected (0-4, 4-8, 8-12 and 8-16 weeks respectively). AHSV types 5, 7 and 9 were not detected by RT-qPCR in any of the weanling groups.

In group I, six weanlings (55%) tested positive for AHSV-1, three (27%) for AHSV-3 and two (18%) for AHSV-4, all within the first 8 weeks. In group III, the other group that received OBP comb1 initially, 5 (45%) tested positive for AHSV-1 and 3 (27%) for AHSV-3, also all within the first 8 weeks. None of the types present in OBP comb2 were detected in any of the weanlings in groups I or III. Three individual weanlings were simultaneously positive for multiple serotypes with one being positive for AHSV-1, AHSV-3 and AHSV-4, one being positive for AHSV-1 and AHSV-3 and one being positive for AHSV-1 and AHSV-4.

In group II, 1 (9%) weanling tested positive for AHSV-2 and 3 (27%) for AHSV-8 within the first 8 weeks after initial immunisation with OBP comb2. One weanling tested positive for AHSV-3 between weeks 8 and 12. In group IV, 3 (27%) tested positive for AHSV-2, 1 (9%) for AHSV-6 and 4 (36%) for AHSV-8 within the first 8 weeks. One (9%) tested positive for both AHSV-1 and AHSV-3 between weeks 12 and 16 (after immunisation with OBP comb1 at week 12).

*Comparisons of the Median Cq Values Over Time in Each of the 4 Groups of Weanling Foals*

There were no significant differences in median Cq values over time between foals in groups I to III, II to III, II to IV or III to IV (Table 5-2), however there was a significant difference in median Cq values over time between group I and group II, ($p$= 0.020) and group I and IV, ($p$=0.008). In both these instances, the foals in group I had significantly lower Cq values and a wider range of values.



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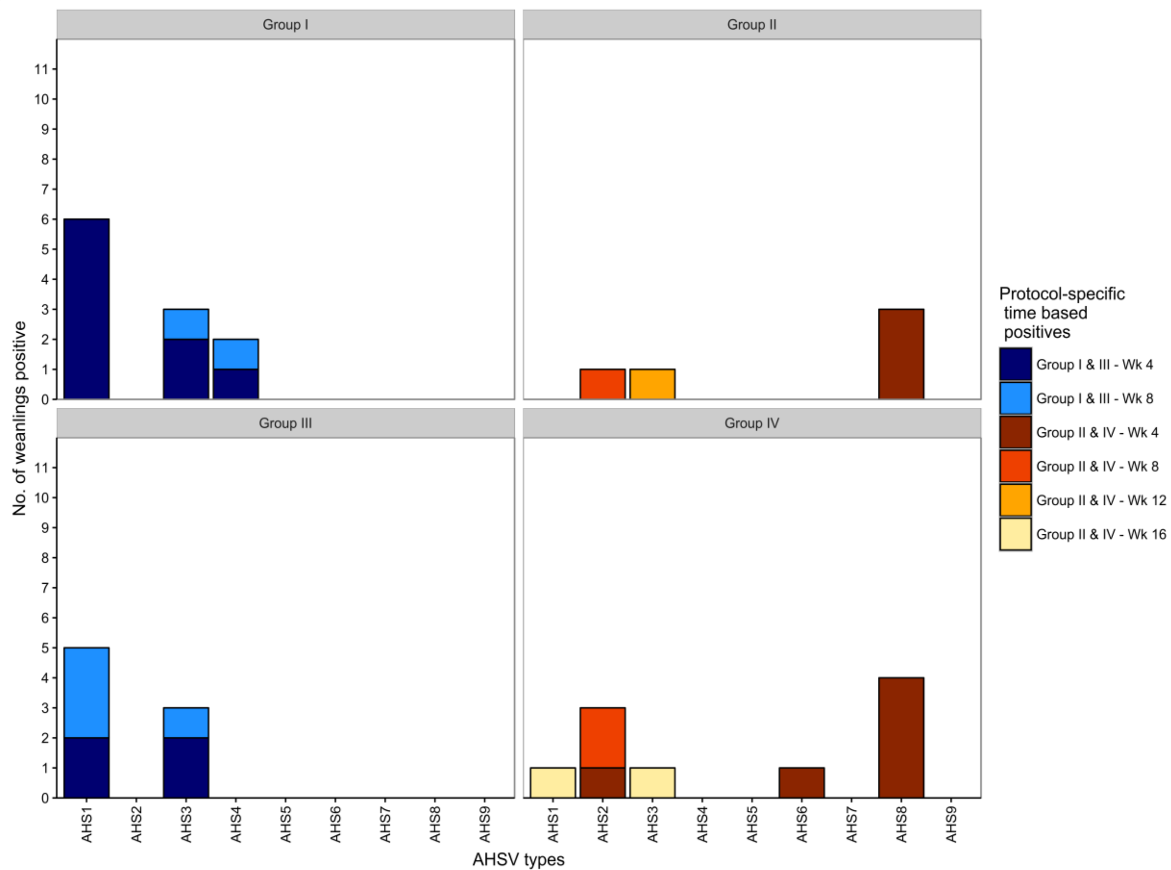


**Figure 5-4: Stacked Bar plots depicting the number of type specific (TS) RT-qPCR positive weanlings for each of the 9 AHSV types on TS-RT-qPCR in each of the 4 weanling groups over time. Plot a) depicts Weanling group I, b) Weanling group II, c) Weanling group III and d) Weanling group IV. Plots a) and c) depicted in blue shades, received OBP comb1 at week 0 and OBP comb2 at weeks 4 and 12 respectively. Plots b) and d) depicted in orange shades, received OBP comb2 at week 0 and OBP comb1 at weeks 4 and 12 respectively. The stacks indicate the number of weanlings that tested positive on TS RT-qPCR per AHSV type in the first 4 week, between weeks 4 and 8, weeks 8 and 12, and weeks 12 and 16. Once a weanling tested positive for a specific type it was not counted for that type again.**

**Table 5-2: A comparison between the different weanling treatment groups using the Wilcoxon Rank-Sum test. The table shows the *p-values* calculated for comparisons between each of the treatment groups. *A *p-value* < 0.05 indicates a significant difference.**

| | Wilcoxon Rank-Sum Test *p-values:* Median Cq value over time | | |
|---|---|---|---|
| | **Group 1** | **Group 2** | **Group 3** |
| **Group 2** | 0.020* | - | - |
| **Group 3** | 0.053 | 0.934 | - |
| **Group 4** | 0.008* | 0.807 | 0.461 |

*GS and TS RT-qPCR Results for OBP comb1 and OBP comb2:*

Results for GS and TS RT-qPCR results for OBP comb1 and OBP comb2 are available in Table 5-3.



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**Table 5-3: GS RT-qPCR results and TS RT-qPCR results for Onderstepoort Biological Products polyvalent OBP comb1 and OBP comb2**

| | Type | AHSV GS RT-qPCR | AHSV TS RT-qPCR | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **1** | **2** | **3** | **4** | **6** | **7** | **8** | **7Vacc** |
| **OBP comb1** | **1** | 17.8 | 21.9 | * | * | * | * | * | * | * |
| | **3** | | * | * | 23.3 | * | * | * | * | * |
| | **4** | | * | * | * | 31.8 | * | * | * | * |
| **OBP comb2** | **2** | 17.3 | * | 25.2 | * | * | * | * | * | * |
| | **6** | | * | * | * | * | 32.0 | * | * | * |
| | **7** | | * | * | * | * | * | * | * | 20.8 |
| | **8** | | * | * | * | * | * | * | 23.4 | * |

The numeric values indicate the Cq value for each type reference sample. * indicates a Cq value of >37, which is considered negative.

*Serum neutralisation tests*

The SNT results for all weanling groups are summarised in Figure 5-5 (and Appendix C). In group I, seroconversion to all types was observed, with the highest prevalence of seroconversion within 8 weeks of vaccination seen for types 1 and 4. A total of 3 weanlings (27%) seroconverted to all 9 types and 2 weanlings did not seroconvert to any of the 9 types. Two individual weanlings only seroconverted to types 3 and 6, respectively, by week 16.

In group II, seroconversion to all types was not observed (type 7 seroconversion was not seen), with highest prevalence of seroconversion within 8 weeks of vaccination seen for types 1 and 6. By week 16 a further six individual weanlings had seroconverted to AHSV-1, 3, 4, 6, 8 and 9, respectively. One weanling in this group seroconverted to 7 of the 9 types and 2 weanlings did not seroconvert to any of the 9 types. One individual only seroconverted to type 1 by week 16.

In group III, the predominant type seroconverted against was type 1 with 9 weanlings seroconverting to this type within 8 weeks after vaccination. This was more marked by week 16 where all 11 weanlings had seroconverted to both type 1 and 6. One weanling in this group seroconverted to 8 of the 9 types.

In group IV, seroconversion to all types was observed in this group by week 16. The predominant type seroconverted against was again AHSV-1. Two weanlings in this group seroconverted to 8 of the 9 types and one weanling did not seroconvert to any of the 9 types.



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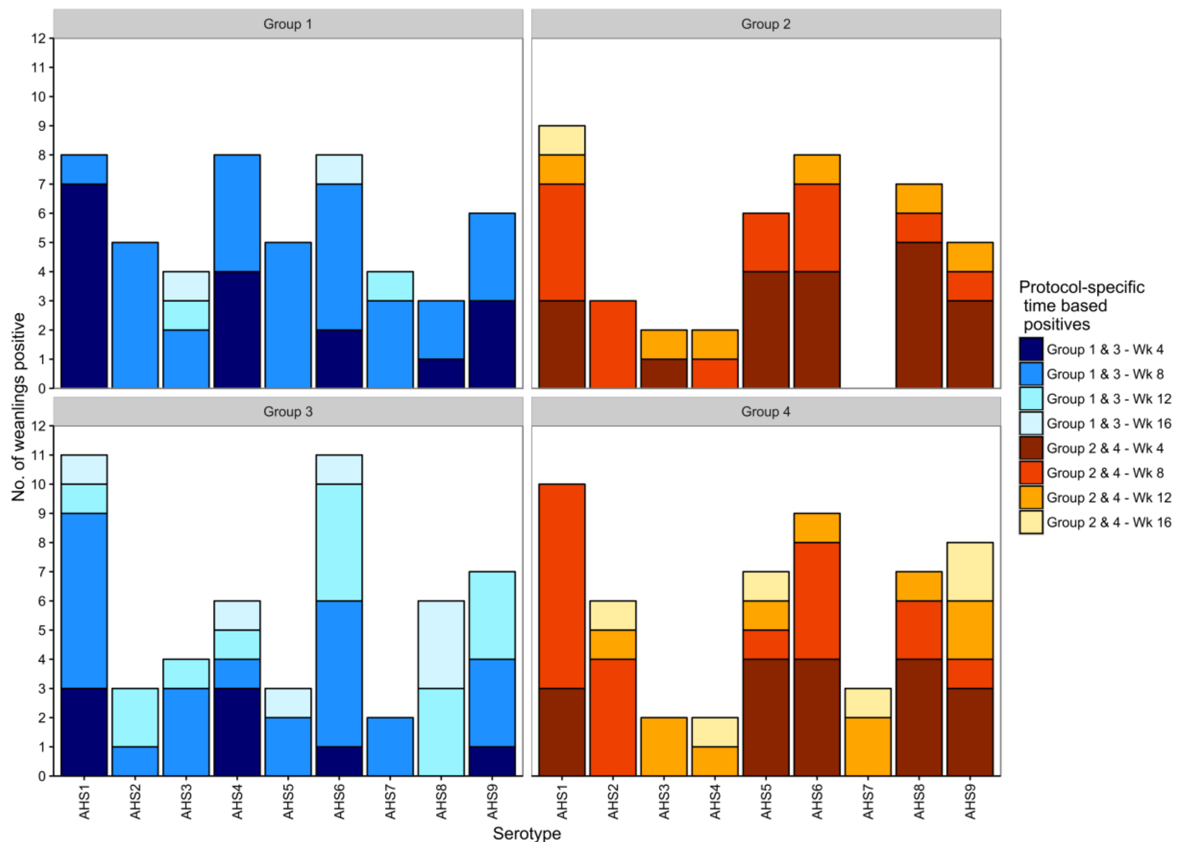


**Figure 5-5: Serological responses of weanlings following immunisation with a polyvalent AHSV-LAV. Stacked Bar plots depicting the number of weanlings that seroconverted (SNT titre > 10) for each of the 9 AHSV types. Plot a) depicts Weanling group I, b) Weanling group II, c) Weanling group III and d) Weanling group IV. Plots a) and b) depicted in blue shades, received OBP comb1 at week 0 and OBP comb2 at weeks 4 and 12 respectively. Plots c) and d) depicted in orange shades, received OBP comb2 at week 0 and OBP comb1 at weeks 4 and 12 respectively. The stacks indicate the number of weanlings that seroconverted per AHSV type in the first 4 week, between weeks 4 and 8, weeks 8 and 12, and weeks 12 and 16.**

*Comparisons of the serological responses for the different weanling Groups*

There were no significant differences in the seroconversion to each of the 9 types of AHSV between the 4 groups of weanlings, as determined using a two way analysis of variance. Thus, there was no significant difference associated with any of the immunization protocols used.

***Yearlings (n=22)***

Results of RT-qPCR and TS RT-qPCR assays performed on samples collected from yearlings following secondary immunization are provided in Table 5-4. Comparison of the number of weanlings positive on GS RT-qPCR following the weanling immunizations to the number of positive yearlings following yearling immunizations showed that substantially fewer animals become positive on RT-qPCR following secondary immunization (Figure 5-6).



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**Table 5-4: A summary of the Group Specific RT-qPCR (GS) and Type Specific RT-qPCR (TS) results over time for each of the yearlings in Weanling group I and II. The Cq value for each of the GS results is given, and the TS result indicating which AHS type was obtained is given below each corresponding positive GS result. N indicates that the Cq value for the GS PCR was > 37. The Minimum Cq per week is given as well as the total number of positive yearlings on GS PCR per week. ND means no AHSV type could be detected. The column highlighted in red indicates the week when OBP comb1 was given (types 1, 3 and 4) and the column highlighted in green indicates the week OBP comb2 (types 2, 6, 7 and 8) was given.**

| Weanling Group | Horse | PCR | Weeks since initial weanling vaccination | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
| I | 3 | GS | 33.8 | N | 34.4 | N | 34.2 | N | 35.4 | 36.9 | N |
| | | TS | | | | | | | | | |
| I | 7 | GS | 34.9 | N | 36.5 | N | N | N | N | N | 34.2 |
| | | TS | | | | | | | | | |
| I | 8 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| I | 14 | GS | 32.4 | 36.9 | 33.0 | N | 35.0 | N | N | N | N |
| | | TS | 3 | | | | | | | | |
| I | 27 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| I | 30 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| I | 38 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| I | 39 | GS | 36.7 | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| I | 45 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| I | 51 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| I | 53 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| II | 1 | GS | N | N | N | N | 35.1 | N | 32.5 | 35.9 | N |
| | | TS | | | | | | | 1 | | |
| II | 9 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| II | 16 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| II | 25 | GS | 37.0 | N | N | N | N | N | N | N | 31.1 |
| | | TS | | | | | | | | | ND |
| II | 28 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| II | 29 | GS | 35.3 | N | 34.8 | N | 36.0 | N | N | N | N |
| | | TS | | | | | | | | | |
| II | 37 | GS | 35.0 | N | 34.1 | 34.2 | 31.9 | 35.5 | 32.7 | 33.8 | 33.1 |
| | | TS | | | | | 1 | | 1 | | |
| II | 41 | GS | N | N | N | N | 35.3 | N | N | N | N |
| | | TS | | | | | | | | | |
| II | 42 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| II | 44 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| II | 48 | GS | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | |
| | Min. Cq | | 32.4 | 36.9 | 33.0 | 34.2 | 31.9 | 35.5 | 32.5 | 33.8 | 31.1 |
| | Total Pos | | 7 | 1 | 5 | 1 | 6 | 1 | 3 | 3 | 3 |



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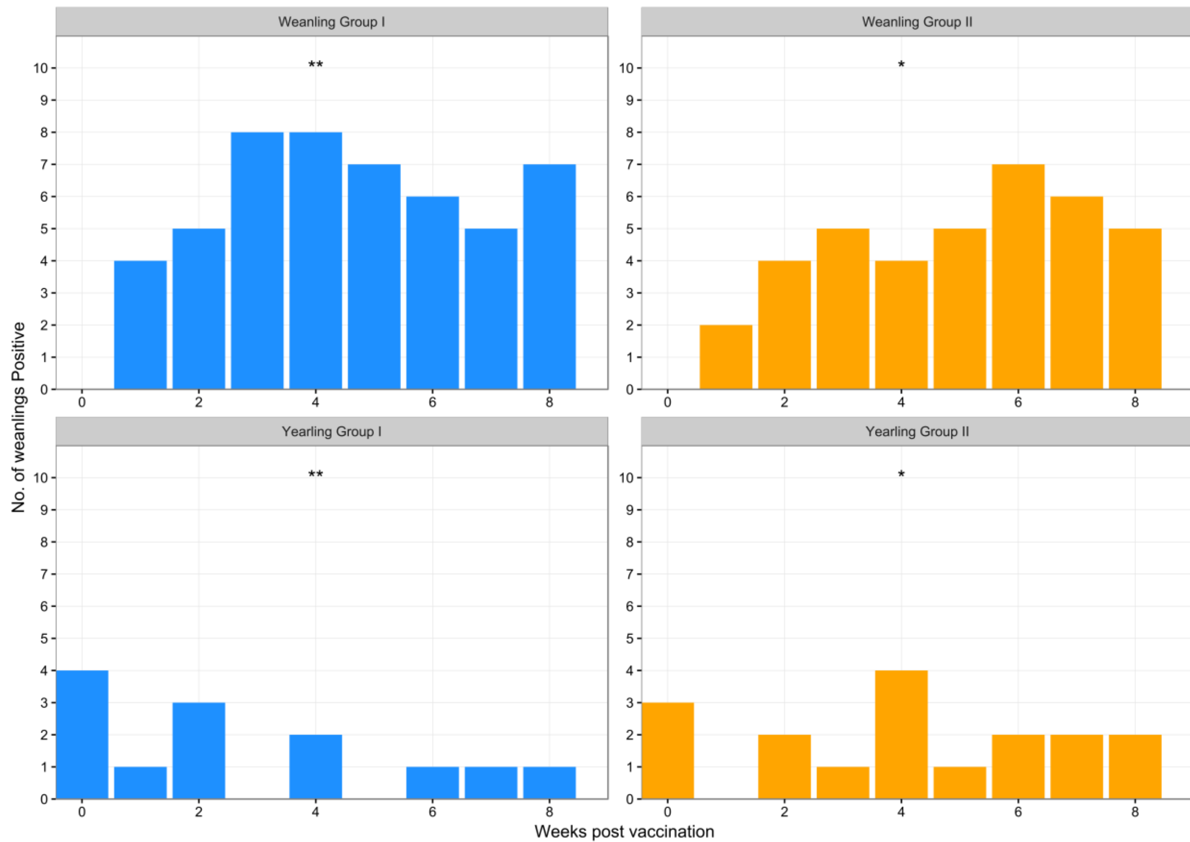


**Figure 5-6: Barplots comparing the total number of positive animals on GS RT-qPCR for Weanling group 1 after the weanling vaccinations at week 0 (a) and yearling vaccinations at week 16 (b) and comparing Weanling group 2 after weanling vaccinations at week 0 (c) and yearling vaccinations at week 16 (d). The x-axis represents the number of weeks after the initial vaccine bottle for each group. Weanling group 1 received OBP comb1 at week 0 followed by OBP comb2 four weeks during their weanling vaccinations. Weanling group 2 received OBP comb2 followed by OBP comb1 four weeks later during their weanling vaccinations. Both groups received OBP comb1 followed by OBP comb2 during their yearling vaccinations at week 16.**

***Mares (n=22)***

All mares tested negative by RT-qPCR following annual booster immunization with AHSV-LAV (Table 5-5).



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**Table 5-5: A summary of the Group Specific RT-qPCR (GS) results over time for each of the mares. Group A consisted of mares that had received 3 to 5 AHS vaccinations in their life (n=7), and Group B, mares that have received 6 of more AHS vaccinations (n=15). N indicates that the Cq value for the GS PCR was > 37. The * indicates that the sample was not tested. The column highlighted in red indicates the week when OBP comb1 was given (types 1, 3 and 4) and the column highlighted in green indicates the week OBP comb2 (types 2, 6, 7 and 8) was given. The number of vaccination previously received for each of the mares is given.**

| Mare Group | No. of Vaccinations Received previously | Horse | Weeks post vaccination | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| B | 12 | 65 | N | N | N | N | N | N | N |
| B | 9 | 66 | N | N | N | * | N | N | N |
| B | 14 | 67 | N | N | N | N | N | N | N |
| A | 5 | 68 | N | N | N | N | N | N | N |
| A | 5 | 69 | N | N | N | N | N | N | N |
| B | 8 | 70 | N | N | N | N | N | N | N |
| A | 5 | 71 | N | N | N | N | N | N | N |
| B | 12 | 72 | N | N | N | N | N | N | N |
| A | 3 | 73 | N | N | N | N | N | N | N |
| A | 5 | 74 | N | N | N | N | N | N | N |
| B | 12 | 75 | N | N | N | N | N | N | N |
| B | 11 | 76 | N | N | N | N | N | N | N |
| B | 6 | 77 | N | N | N | N | N | N | N |
| B | 14 | 78 | N | N | N | N | N | N | N |
| B | 12 | 79 | N | N | N | N | N | N | N |
| A | 3 | 80 | N | N | N | N | N | N | N |
| B | 9 | 81 | N | N | N | N | N | N | N |
| B | 12 | 82 | N | N | N | N | N | N | N |
| B | 8 | 83 | N | N | N | N | N | N | N |
| B | 9 | 84 | N | N | N | N | N | N | N |
| B | 10 | 85 | N | N | N | N | N | N | N |
| A | 4 | 86 | N | N | N | * | N | N | N |



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## 5.5 Discussion

The goal of this study was to evaluate the potential impact of RNAemia as a consequence of vaccination on AHSV surveillance that utilizes RT-qPCR assays. Amongst the weanlings immunized with the commercial polyvalent AHSV-LAV, the Cq values were lowest amongst weanlings that received OBP comb1 initially, especially those that received OBP comb2 4 weeks later (group I). Individual weanlings had Cq values as low as 22.3, which is equivalent to that encountered in field and experimental cases of AHS *(56, 176)*. In addition to complicating surveillance, these low Cq values in vaccinated animals likely correlate to high level viraemia and raise concerns regarding the potential risk of transmission of vaccine virus to vector *Culicoides spp.* There was marked variation between individual animals within each group, with some weanlings having Cq values in the low 20s and others remaining negative throughout.

The highest number of AHSV-RNA positive animals per week was reached between weeks 4 and 6 after initial immunization in all groups of weanlings, with the highest numbers obtained in those that received OBP comb1 initially (groups I and III). The weanlings in group I remained positive after immunization longer than those in the other groups, with Cq values often <30. This finding coupled with the detection of multiple AHSV types in the same sample would increase the risk of genome segment reassortment, a feature well described for segmented viruses and that has been reported between Orbivirus strains during infection of vertebrate hosts, insect vectors and cell cultures *(123, 145, 149)*. In this study, animals in groups I and III received the different vaccine combinations at an interval of four weeks between the doses and it is presumed that the risk of reassortment would increase if the interval between immunizations was further decreased. The interval between combinations is recommended to be at least 3 weeks by the manufacturer.

Comparison of the results obtained with GS RT-qPCR in weanling foals and yearling animals clearly confirms a markedly different outcome. Specifically, weanlings were approximately twice as likely to be RNA-positive after secondary immunization as were yearlings and none of the AHSV types from OBP comb2 were detected in the yearlings after secondary immunization. The risk of RNAemia is likely reduced even further after multiple immunizations, as the repeatedly immunized mares all remained negative on GS RT-qPCR.

A number of weanlings tested positive on GS RT-qPCR but no type could be determined by TS RT-qPCR. These discrepancies likely reflect false negative data from the TS RT-qPCR assays as they are only qualitative tests, and have not been validated for use as quantitative



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tests. Furthermore, if low concentrations of nucleic acid from multiple AHSV types are present in a given sample, the sum of common (group-specific) genes may be sufficient to be detected by GS RT-qPCR whereas the amount present from each separate type may be below the detection thresholds of individual TS RT-qPCR assays.

The serological data obtained by SNT in this study is consistent with results of other recent studies *(32, 171)*. Marked variation in SNT titres to individual AHSV types between weanling animals was observed. In the current study, more weanlings consistently seroconverted to AHSV-1 across all the groups than any other type, with AHSV-6 also being well represented in the response to vaccination. Serological responses to AHSV-3 and AHSV-7 were consistently weakest across all four groups. The results of our study for AHSV-4 are in contrast to those reported by Von Teichman et al *(172)* who reported that AHSV-4 was the most immunogenic type. The duration of neutralizing antibody was often transient after immunization as has been reported previously *(32, 171)*. Currently, it is uncertain what this means in terms of protective immunity and this warrants further investigation.

The detection of specific AHSV types by TS RT-qPCR assays was not consistently predictive of an enhanced serological response to that specific type in individual animals. For example, whereas a high number of weanlings in group I tested positive for AHSV-1 by TS RT-qPCR n=6) but not group IV (n= 0), paradoxically more animals in group IV seroconverted to AHSV-1 than those in Group I (10 and 8, respectively). Furthermore, although no weanlings tested positive for AHSV-6 on TS RT-qPCR in Groups I, II or III, a high number of weanlings seroconverted to AHSV-6 in all three groups.

In summary, AHS is a fatal disease in susceptible horses and immunization is currently widely used to control the disease in endemic areas. In this study, the level of AHSV nucleic acid present and the AHSV type involved in weanling foals following immunization was evaluated and compared using GS and TS RT-qPCR assays The animals' serological responses were then analysed using the SNT for each of the 9 types. The results of this study show that although immunisation of naive horses results in RNAemia, administration of OBP comb2 before OBP comb1 reduces RNAemia with fewer animals becoming positive on GS RT-qPCR. Significantly, there was no apparent difference in type specific SNT responses when OBP comb2 was given before OBP comb1. This study also supports the practice of waiting for a longer period than the suggested minimum of at least 3 weeks between the two combinations. A period of six or more weeks between bottles may be safer, as the peak in Cq values and the highest number of weanlings likely to be positive occurred between week 0 and 6 post-immunization.



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## 5.6 Conclusion

This study supports the scenario that the current commercial AHSV-LAV in South Africa is a potential source of virus for transmission by AHS vectors. Furthermore, use of this AHSV-LAV can potentially complicate surveillance because of the high levels of viral RNA that circulate in immunized horses. The risk of viraemia is further complicated by the presence of multiple AHSV types potentially multiplying concurrently in the host cell, increasing the risk of reassortment. There is some risk, therefore, in the use of an AHSV-LAV for protection in an AHS-free zone or country. If the vaccine must be used in these areas then it should be limited to the period of low vector activity, logically in combination with vector protection in the form of stabling, insecticides and repellents.



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# CHAPTER 6

## African horse sickness caused by genome reassortment and reversion to virulence of live, attenuated vaccine viruses, South Africa, 2004 - 2014

### 6.1 Abstract

African horse sickness (AHS) is a devastating haemorrhagic viral fever that currently affects horses and other equine populations throughout much of sub-Saharan Africa. Given its significance to the international trade and movement of horses, AHS is the only equine disease for which the World Organisation for Animal Health (OIE) has introduced specific guidelines so that member countries can seek official recognition of disease-free status. To that end, South Africa has since 1997 maintained an AHS Controlled Area in a historically AHS-free portion of the Western Cape Province. Despite these control efforts, sporadic outbreaks of AHS have continued to occur within the AHS Controlled Area. In an effort to determine the source of the viruses responsible for some of these outbreaks, the whole genome sequences of 39 AHS virus (AHSV) isolates from field AHS cases were compared. The data confirm that the viruses responsible for outbreaks in 2004 and 2011 were likely virulent revertants derived from the AHSV type 1 (AHSV-1) live, attenuated vaccine (LAV). Furthermore, viruses isolated during outbreaks in both 2004 and 2014 were reassortants with genome segments derived from all three of the AHSV types (1, 3 and 4) contained in the commercial trivalent AHSV-LAV that is used widely throughout South Africa. These data indicate that despite highly effective protection of vaccinated horses, the use of this polyvalent AHSV-LAV may, paradoxically, place susceptible horses at risk of being infected with AHSV.

### 6.2 Introduction

African horse sickness (AHS) is a severe, often fatal disease of equids that is caused by AHS virus (AHSV), a member of the genus *Orbivirus* in the family *Reoviridae (30, 184)*. The virus is transmitted to horses by biting midges in the genus *Culicoides (36, 169).* Although AHS currently occurs only in sub-Saharan Africa, various species of *Culicoides* midges occur throughout virtually the entire inhabited world and there is, therefore, concern that AHSV could spread into areas that are currently free of the virus *(58, 97, 99, 184)*. For example, AHSV spread during the 1960s into the Middle East, Eastern Europe, and the Indian subcontinent, and in the 1980's into the Iberian Peninsula and North Africa *(83, 95, 140)*. Furthermore, the global range of related *Culicoides*-transmitted orbiviruses such as bluetongue virus has expanded recently, likely in part as a consequence of the impact of climate change on midge



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vectors of the virus *(98, 136)*. In AHSV-endemic temperate regions such as those occurring throughout much of South Africa, AHS is most prevalent during the time of year, and in areas where vector midges are most abundant; i.e. during the late summer in regions with summer rainfall *(30, 60)*.

Efforts to prevent the catastrophic impact of AHS began soon after the determination of its viral etiology in 1900, when at the time it was only the second animal virus ever described *(117, 162)*. Efficacious AHSV live, attenuated vaccines (LAV) were first developed in South Africa using viruses passaged in the brains of mice *(6)* and, subsequently, cell culture *(45, 79)*. Presently, a polyvalent AHSV-LAV produced by Onderstepoort Biological Products (OBP) Ltd that provides broad-protection against all nine AHSV types *(45, 171)* is used widely in South Africa and adjacent countries. This vaccine is supplied in two vials, each of which contains different combinations of AHSV types: OBP comb1 is trivalent and contains types 1, 3 and 4 whereas OBP comb2 is tetravalent and contains types 2, 6, 7 and 8 *(32, 171)*. Heterologous immunity between types provides immunity to the two AHSV types (5 and 9) that are not included in the vaccine.

Given its adverse impact on the international trade and movement of equids, AHS is the only equine disease for which the World Organisation for Animal Health (Office Internationale des Epizooties [OIE]) has "Official Recognition Status". Specifically, because AHS is a high impact disease with the documented potential for trans-boundary spread, veterinary administrations of the 180 OIE Member Countries are required to have specific, legally enforceable AHS control measures in place, and are required to immediately notify the OIE of any change to their AHS status *(180)*. The Western Cape Province of South Africa which sits at the southern tip of the African continent has historically been free from AHS, and for this reason, a legislatively defined "AHS controlled area" (CA) was created there in 1997 to facilitate trade and movement of horses from South Africa. Within this area are an AHS free zone (FZ), consisting of the Cape Town metropolis, an AHS surveillance zone (SZ) surrounding the FZ, and an outermost AHS protection zone (PZ; Figure 6-1) *(157)*. Movement of equids into and between these zones is strictly controlled. Vaccination with the polyvalent AHSV-LAV in the SZ and FZ is only allowed with permission from the veterinary authority, and since 2015 permission is only granted during the period of low vector activity (winter months – 1 June to 31 October).

Since its creation in 1997, there have been six outbreaks of AHS in the AHS CA that have been reported to the OIE (1999, 2004, 2006, 2011, 2013 and 2014) *(54-56, 138, 154, 155)*. Prior to the 2014 outbreak it was assumed that these outbreaks were all due to the illegal movement of viraemic animals into the CA, even though the actual source was only established for two of these outbreaks: a type 7 virus for the 1999 outbreak in the Stellenbosch



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region and a type 5 virus for the 2006 outbreak in the Robertson region (Figure 6-1). The illegal movement of horses into the AHS CA was confirmed after the 1999 outbreak, one of which was viraemic and subsequently developed clinical AHS. The 2006 outbreak was caused by AHSV-5, which is not included in the commercially available polyvalent AHSV-LAV, and was traced to the illegal movement of horses into the AHS PZ from Johannesburg during a period of active AHSV circulation *(181, 182)*. Since the source of the viruses responsible for the other outbreaks was never established, the goal of the current study was to further characterize the epidemiology of AHS outbreaks in the South African AHS CA by: 1) whole genome sequencing of representative viruses from individual outbreaks (2004, 2011, 2014), 2) phylogenetic comparison of viral genome sequences derived from these outbreaks with those of the polyvalent AHSV-LAV, 3) analysis of outbreak viruses for genome segment reassortment, 4) analysis of single nucleotide variants (SNVs) associated with attenuation of AHSV-LAV to investigate the possibility that vaccine-derived viruses may have reverted to virulence in the field, 5) correlation of epidemiological and clinical findings with molecular findings, and 6) confirmation of the source of the virus strains responsible for the 2004, 2011 and 2014 outbreaks of AHS in the AHS CA of the Western Cape Province.



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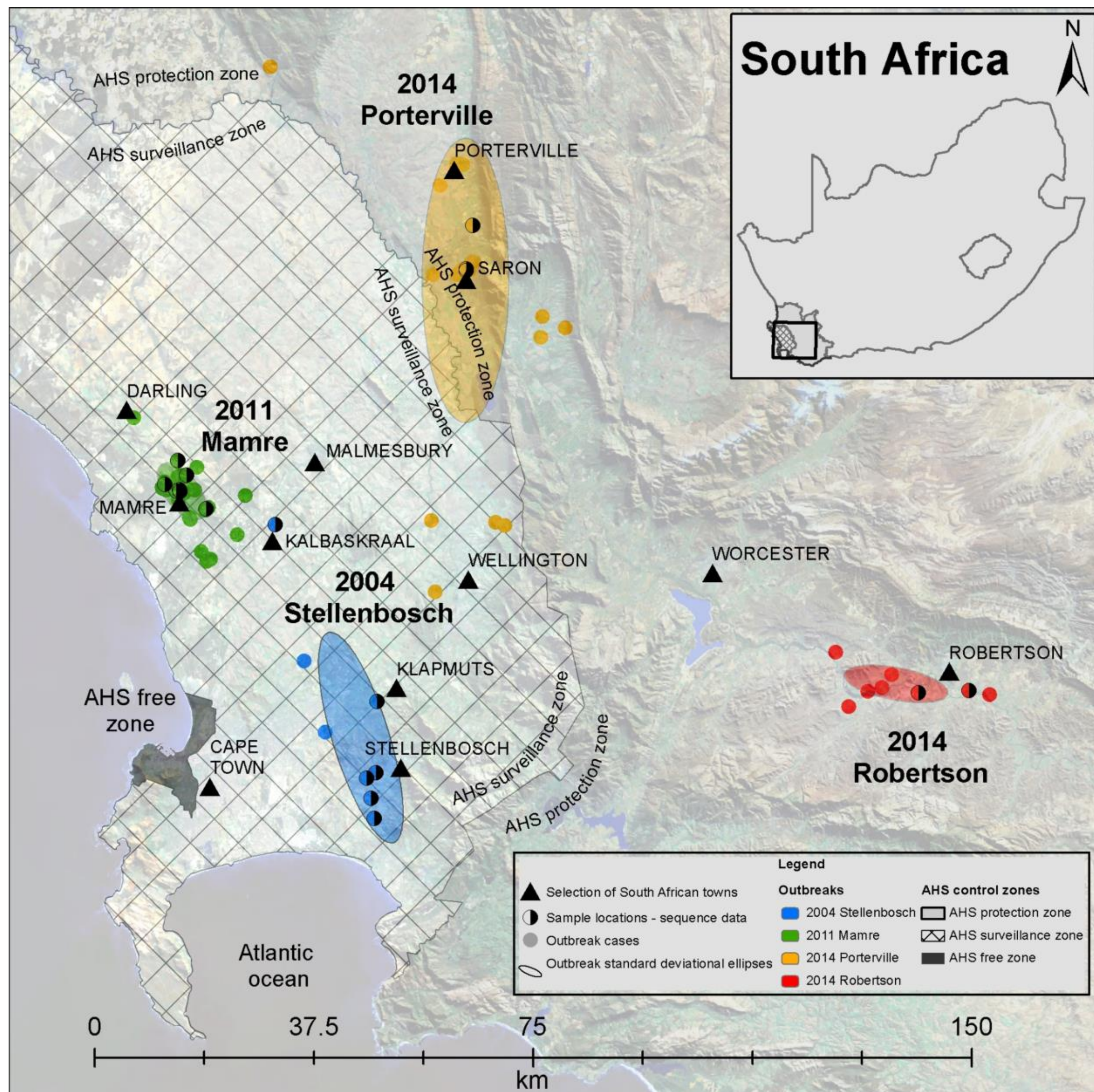


**Figure 6-1: Map showing African horse sickness (AHS) outbreaks included in this study. Map of the AHS controlled area of the Western Cape Province of South Africa showing the spatial distribution of each of the AHS virus type 1 outbreaks that have occurred in this area since 1997. The AHS free, surveillance and protection zones are also shown. Individual confirmed cases of AHS are indicated by the solid dots. The directional distribution of each outbreak is indicated using standard deviational ellipses.**

## 6.3 Materials and Methods

### *Virus isolates*

Complete genomes were sequenced from 55 AHSV isolates collected between 1961 and 2014, including: 39 field isolates of AHSV-1 from horses during the 2004 Stellenbosch (n=16), 2011 Mamre (n=7), 2014 Porterville (n=14) and 2014 Robertson (n=2) outbreaks of AHS in the Western Cape Province of South Africa (Figure 6-1) seven AHSV-LAV strains of types 1, 2, 3, 4, 6, 7 and 8 and Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI)



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Laboratory Reference strains for each of the nine AHSV types. Each of these virus isolates was included in the AHS genome sequencing Bioproject (**http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA271179**) and was identified by a unique virus strain name. The details of the strain names, sample source, the Biosample and GenBank accession numbers of the whole genome sequences of each of the 39 field isolates and 16 AHSV-LAV and laboratory strains of AHSV are provided in Appendix D.

***RNA extraction, identification and typing***

Individual viruses of each type included in the polyvalent AHSV-LAV were independently isolated using plaque selection on Vero cells in the presence of heterologous antibody to the other virus types contained in the trivalent and tetravalent vaccine preparations, as previously described *(66, 67)*. AHSV dsRNA derived from all strains of AHSV that were evaluated (i.e. field, reference and AHSV-LAV viruses) was extracted from virus-infected cells using TRIzol reagent (Life Technologies, Johannesburg, South Africa). Identification and typing of AHSV isolates was done by group-specific real-time reverse transcriptase polymerase chain reaction (GS RT-qPCR) assays *(61)* and type specific (TS) RT-qPCRs targeting conserved epitopes within the gene encoding VP2 *(177)*.

***Genome sequencing and assembly***

Sequencing templates were prepared using sequence-independent whole-genome RT-PCR amplification *(132)*. PCR amplicons were sequenced on an Illumina MiSeq sequencer (Inqaba Biotechnical Industries [Pty] Ltd., Pretoria, South Africa) using the Nextera XT DNA sample preparation kit and 300-bp paired-end V3 Illumina chemistry. Illumina sequence reads were analyzed using Geneious version 9 (**http://www.geneious.com**) *(89)*. A combination of *de novo* assembly followed by mapping was used to obtain the full-length consensus genome sequences of each virus strain.

***Phylogenetic analysis***

Sequences of the concatenated whole virus genomes and individual genome segments were aligned using MAFFT *(87)* implemented within Geneious version 9 (**http://www.geneious.com**) *(89)*. Once aligned, the Smart Model Selection (SMS) program implemented in PhyML Version 3 (**http://www.atgc-montpellier.fr/phyml/**) *(59)* was used to identify the evolutionary models that best fit the individual sequence datasets using the corrected Akaike Information Criterion (AIC). The parameters from these models were used to construct maximum likelihood trees using PhyML Version 3 *(59)* implemented within Geneious



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version 9 (http://www.geneious.com) *(89)* with 1000 bootstrap replicates to estimate branch support.

***Genotype group analysis***

RAMI (http://mbio-serv2.mbioekol.lu.se/rami.html) *(129)* was used to analyse the concatenated whole genome sequence maximum likelihood (ML) tree to genetically (and not evolutionarily) classify the sequences into genotype groups based on patristic distances (P, i.e. branch lengths separating the isolates within the ML). RAMI was run with the patristic distance threshold set to 0.000459. This threshold was selected because it was intended to differentiate between genome sequences that differed from one another by as few as 16 nucleotide variants.

***Reassortment analysis***

Recombination Detection Program v.4.63 (RDP4) *(106)* was used with default settings, except that we invoked the "scan for reassortment and recombination" setting to identify any reassortment between the gene segments of AHSV-LAV vaccine strains of AHSV types 1, 3 and 4 and the 39 field isolate strains included in this study. Reassortment events detected by any of the eight different recombination detection methods implemented in RDP4 (the RDP, MAXCHI and GENECONV methods in primary scanning mode, the BURT, Bootscan, CHIMAERA, SisScan, and 3SEQ methods in secondary scanning mode, each with a Bonferroni corrected p-value cutoff of 0.05) were considered to represent evidence of reassortment.

***Non-synonymous single nucleotide variants***

Consensus concatenated whole virus genomes from the viruses, 1/Lab/ZAF/62/OVI-HS29/62 and 1/Lab/ZAF/98/OBP-116, and the 39 field isolate strains were aligned using MAFFT *(87)* and were analysed using the "Find Variations/SNPs" function in Geneious version 9 (http://www.geneious.com) *(89)* with the "Find non-synonymous polymorphisms only" option enabled. The non-synonymous single nucleotide variants (nsSNVs) in these sequences were compared with the nsSNVs previously associated with attenuation of AHSV-1 *(132)*.



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## 6.4 Results

### *Phylogenetic and genotype group analysis*

Concatenated genome segments of 55 AHSV genomes (APPENDIX C) were used to construct a ML phylogenetic tree incorporating best fit substitution models (Table 6-1) in order to infer degrees of genetic relatedness (as opposed to evolutionary relatedness) between them (Figure 6-2). Genotype group analysis of patristic distances inferred from this ML tree using RAMI *(129)* indicated that the AHSV strains isolated during the 2004, 2011 and 2014 outbreaks of AHS in the CA segregate into three, one and two unique groups, respectively. These genotype groups are identified as 1a, 1b, and 1c for the 2004 Stellenbosch outbreak, 2 for the 2011 Mamre outbreak, and 3a and 3b for the 2014 Porterville and Robertson outbreaks, respectively. For the Stellenbosch outbreak viruses, genotype group 1a includes four viruses that group closely with the AHSV-1-LAV strain, 1/Lab/ZAF/98/OBP-116, genotype group 1b includes eleven viruses that are also closely related to 1/Lab/ZAF/98/OBP-116, and genotype group 1c includes a single virus that segregates between 1/Lab/ZAF/98/OBP-116 and 4/Lab/ZAF/98/OBP-116. The Mamre outbreak viruses in genotype group 2 consist of seven viruses that are all closely related to 1/Lab/ZAF/98/OBP-116. For the 2014 outbreak viruses, genotype group 3a includes 14 viruses that were all isolated from AHS cases in the Porterville area, whereas genotype group 3b includes two viruses that were isolated from cases in the Robertson area with both groups of viruses being closely related to 1/Lab/ZAF/98/OBP-116. The RAMI indices describing the different genotype groups are provided in Table 6-2.

**Table 6-1: Summary of results of best fit substitution models obtained using PhyML-SMS (Smart Model Selection) with the Akaike Information Criterion for the concatenated alignments of the complete AHSV genomes and individual AHSV gene segments of the strains included in this study.**

| Gene | Model | Proportion Invariable | Rate Category | Gamma shape |
|---|---|---|---|---|
| **VP1** | GTR +G6 | 0 | 6 | 0.261 |
| **VP2** | GTR +G6 +I | 0.186 | 6 | 1.196 |
| **VP3** | GTR +G6 | 0 | 6 | 0.157 |
| **VP4** | GTR +G6 +I | 0.44 | 6 | 0.874 |
| **VP5** | GTR +G6 +I | 0.356 | 6 | 0.599 |
| **VP6** | GTR +G6 | 0 | 6 | 0.459 |
| **VP7** | GTR +G6 +I | 0.616 | 6 | 1.379 |
| **NS1** | GTR +G6 +I | 0.606 | 6 | 1.184 |
| **NS2** | GTR | 0 | 1 | |
| **NS3** | GTR +G6 | 0 | 6 | 0.652 |



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**Table 6-2: RAMI indices describing the microdiverse clusters identified in AHSV1 isolates from the 2004, 2011 and 2014 outbreaks of AHS in the Western Cape Province of South Africa.**

| Cluster | Abundance | $X_{distance}$ | $X_{depth, nearest}$ | $X_{depth, deepest}$ | $Y_{distance}$ | $Y_{depth, nearest}$ | $Y_{depth, deepest}$ |
|---|---|---|---|---|---|---|---|
| **1a** | 4 | 0.00011 | 0.000055 | 0.000055 | 0.120843 | 0.000274 | 0.17623 |
| **1b** | 11 | 0.000067 | 0.000034 | 0.000034 | 0.113798 | 0.004972 | 0.165405 |
| **1c** | 1 | 0 | 0 | 0 | - | - | - |
| **2** | 7 | 0.00033 | 0.000102 | 0.000187 | 0.120925 | 0.00036 | 0.176316 |
| **3a** | 14 | 0.000121 | 0.000041 | 0.000062 | 0.114587 | 0.000173 | 0.166889 |
| **3b** | 2 | 0.000459 | 0.000229 | 0.000229 | 0.114779 | 0.000375 | 0.167091 |
| **Other** | 1 | 0 | 0 | 0 | - | - | - |
| **Average** | 6.5 | 0.0002174 | 0.0000922 | 0.0001134 | 0.1169864 | 0.0012308 | 0.1703862 |

Given that reassortment is a major feature of orbivirus evolution *(121, 148, 149, 160)* and had, therefore, likely obscured the evolutionary relationships of the 55 AHSV isolates in the full-genome AHSV ML tree, evolutionary relationships between the sequences were further explored by constructing separate ML trees for each of the VP1, VP2, VP3, VP4, VP5, VP6, VP7, NS1, NS2 and NS3 encoding genome segments Figure 6-3 to Figure 6-12. For the segments encoding VP2, VP3, VP6, NS1 and NS2 (Figure 6-4, Figure 6-5, Figure 6-8, Figure 6-10 and Figure 6-11), viruses included in genotype groups 1a, 1b, 1c, 2, 3a and 3b all group, with high degrees of associated bootstrap support, together with the AHSV-1-LAV strain, 1/Lab/ZAF/98/OBP-116. For the segments encoding VP1, VP4 and VP7 (Figure 6-3, Figure 6-6 and Figure 6-9), the viruses included in genotype groups 1a, 2, 3a and 3b also segregate with 1/Lab/ZAF/98/OBP-116 whereas those in genotype groups 1b and 1c segregate with the AHSV-3-LAV strain, 3/Lab/ZAF/98/OBP-116. For the gene encoding VP5, viruses included in all genotype groups except 1c segregate with 1/Lab/ZAF/98/OBP-116 whereas those in genotype group 1c segregate with the AHSV-4-LAV strain, 4/Lab/ZAF/98/OBP-116 (Figure 6-7). For genes encoding NS3, viruses included in genotype groups 1a and 2 segregate with 1/Lab/ZAF/98/OBP-116 whereas those included in the remaining genotype groups segregate with 4/Lab/ZAF/98/OBP-116 (Figure 6-12). Collectively, these data confirm that all ten gene segments of the viruses included in genotype groups 1a and 2 are likely derived from a most recent common ancestor closely resembling 1/Lab/ZAF/98/OBP-116; the viruses included in genotype groups 1b and 1c are likely reassortants derived from parental viruses very closely resembling 1/Lab/ZAF/98/OBP-116 and 3/Lab/ZAF/98/OBP-116 and/or 4/Lab/ZAF/98/OBP-116; and viruses in genotype groups 3a and 3b are likely reassortants derived from parental viruses very closely resembling 1/Lab/ZAF/98/OBP-116 and 4/Lab/ZAF/98/OBP-116 (Figure 6-13).



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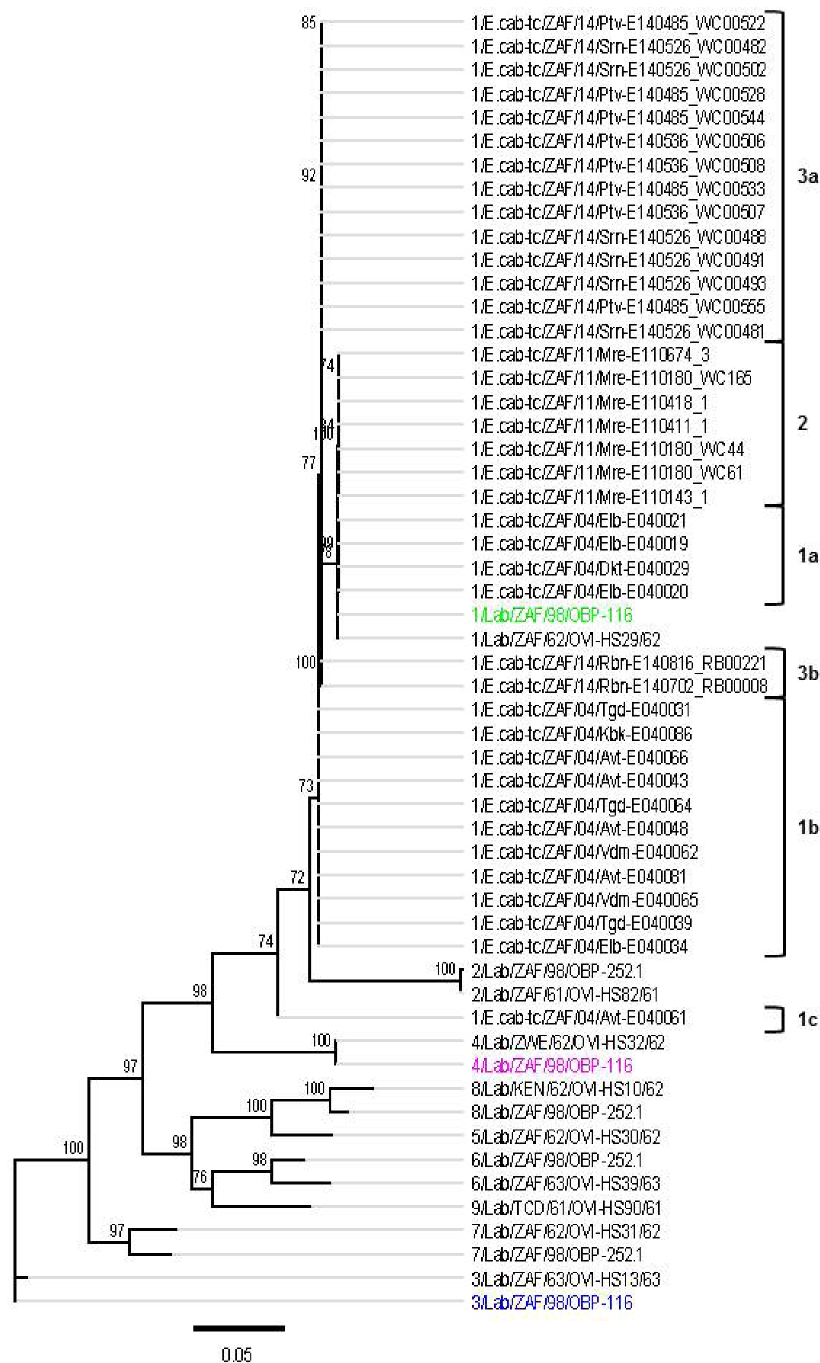


**Figure 6-2: Whole genome phylogeny of African horse sickness viruses (AHSV). Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated whole genome nucleotide sequences of viruses from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS CA to the AHSV live, attenuated vaccine and reference viruses. Branches are scaled to represent numbers of inferred nucleotide differences per site with the scale bar at the bottom of the tree indicating genetic distance. Branches supported by full maximum likelihood bootstrap values >70% are indicated. Genotype groups are indicated with brackets and group names to the right of the tree.**



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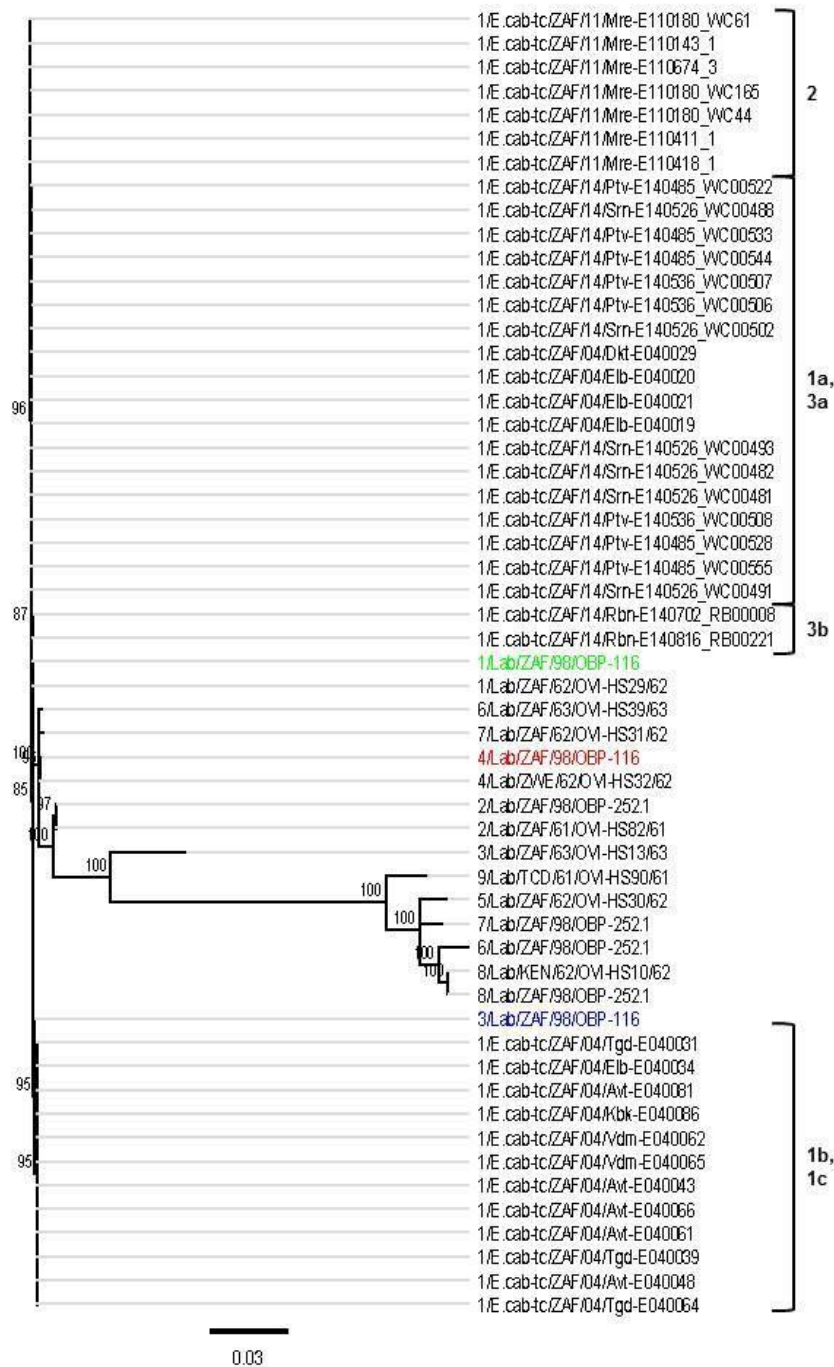


**Figure 6-3: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP1 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. Branches are scaled to represent numbers of inferred nucleotide differences per site with the scale bar at the bottom of the tree indicating genetic distance. Branches supported by full maximum likelihood bootstrap values >70% are indicated. Genotype groups are indicated with brackets and group names to the right of the tree.**



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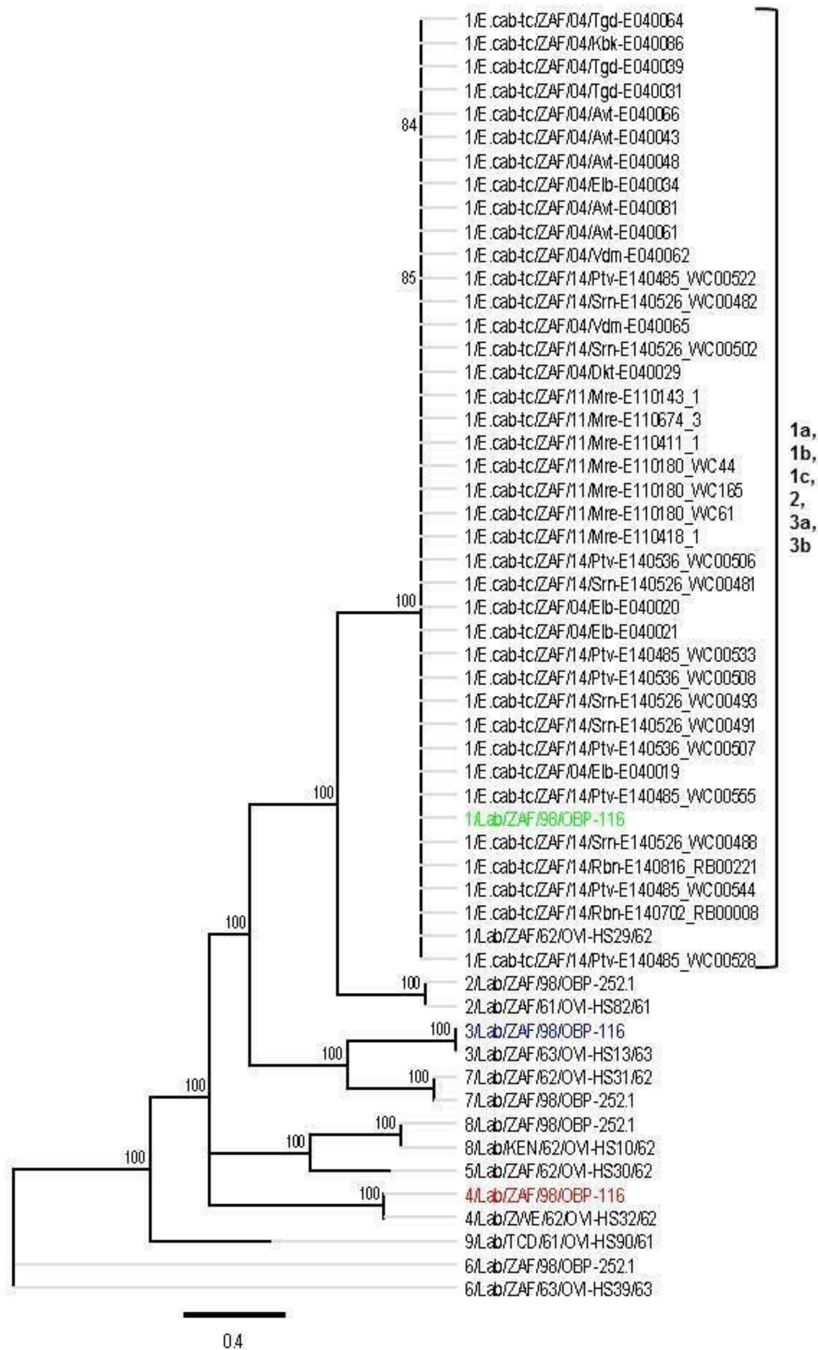


**Figure 6-4: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP2 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details.**



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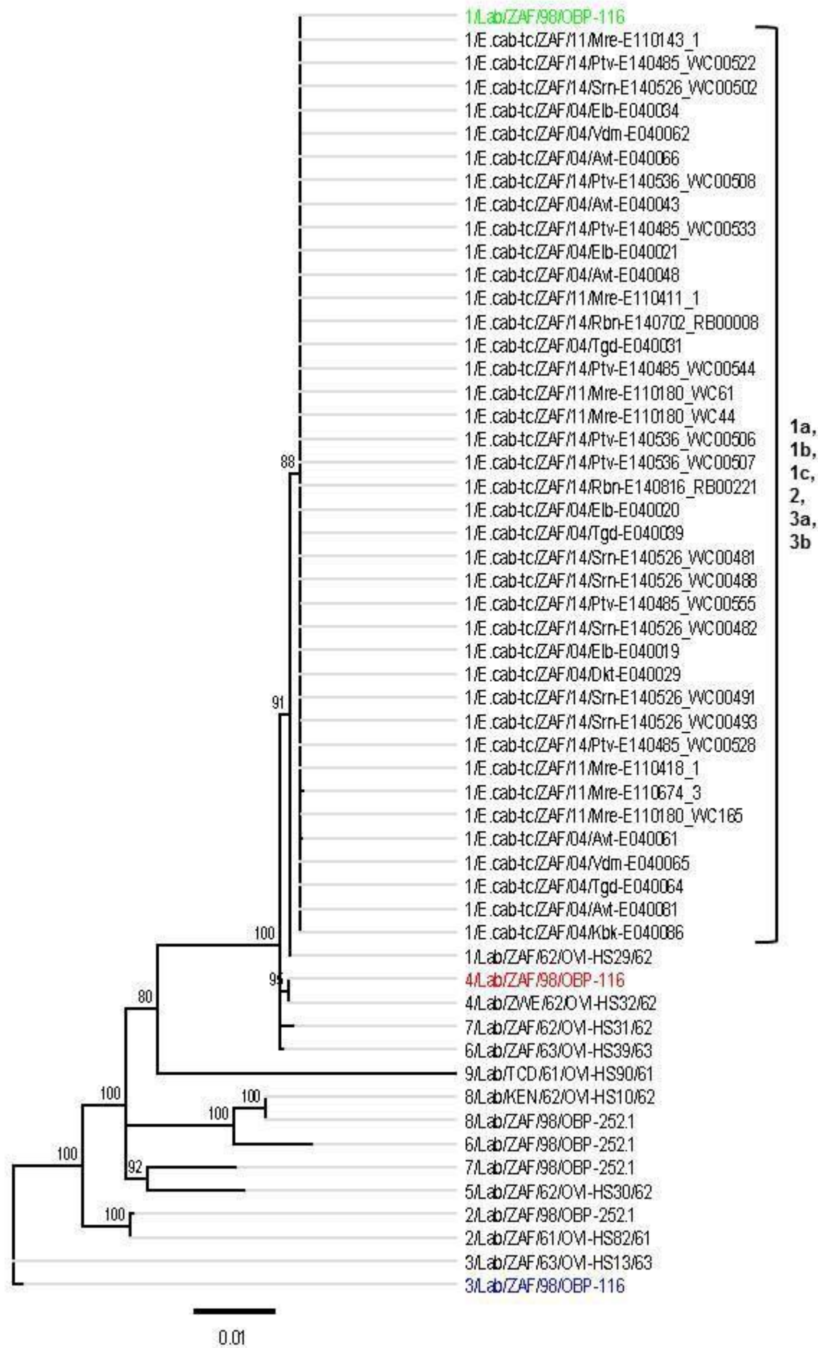


**Figure 6-5: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP3 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details.**



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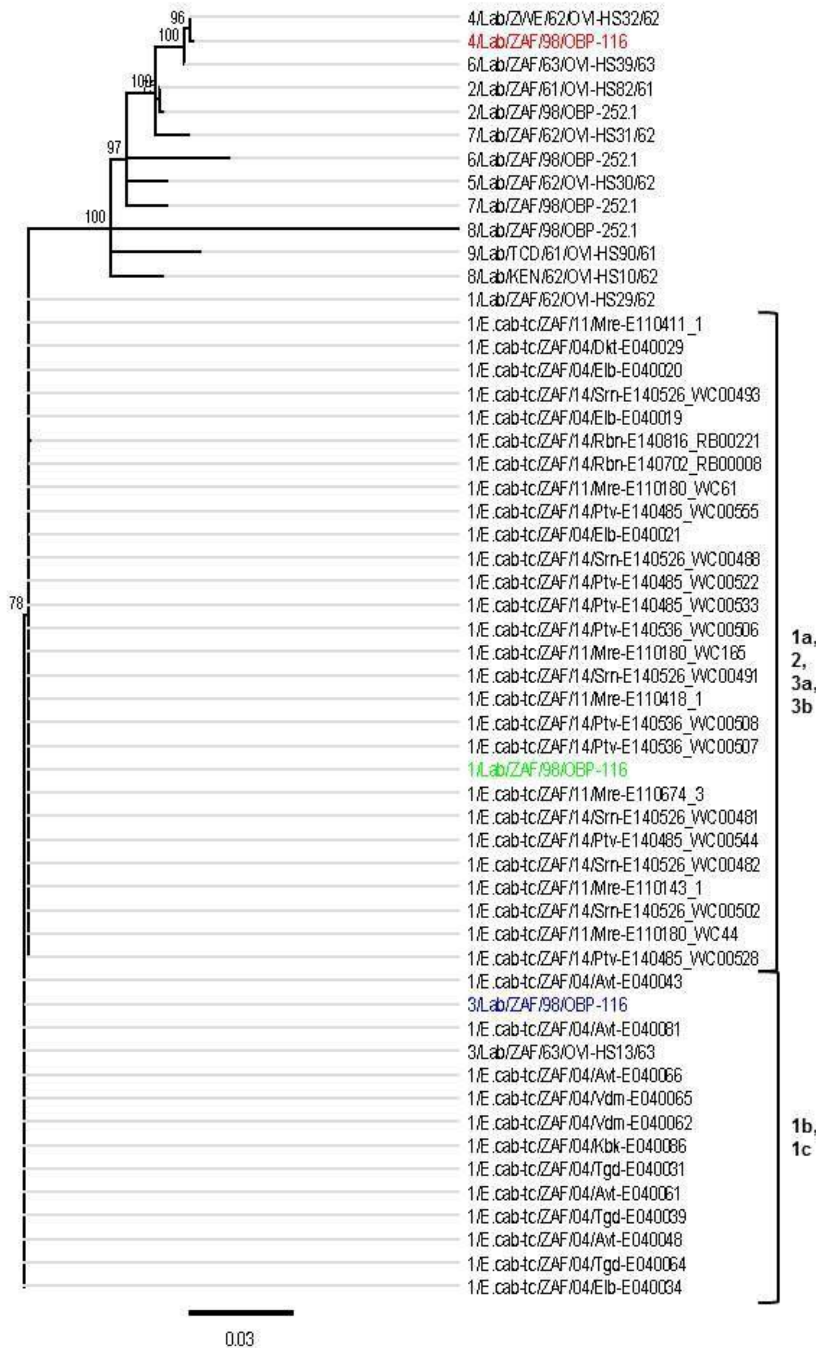


**Figure 6-6: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP4 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details.**



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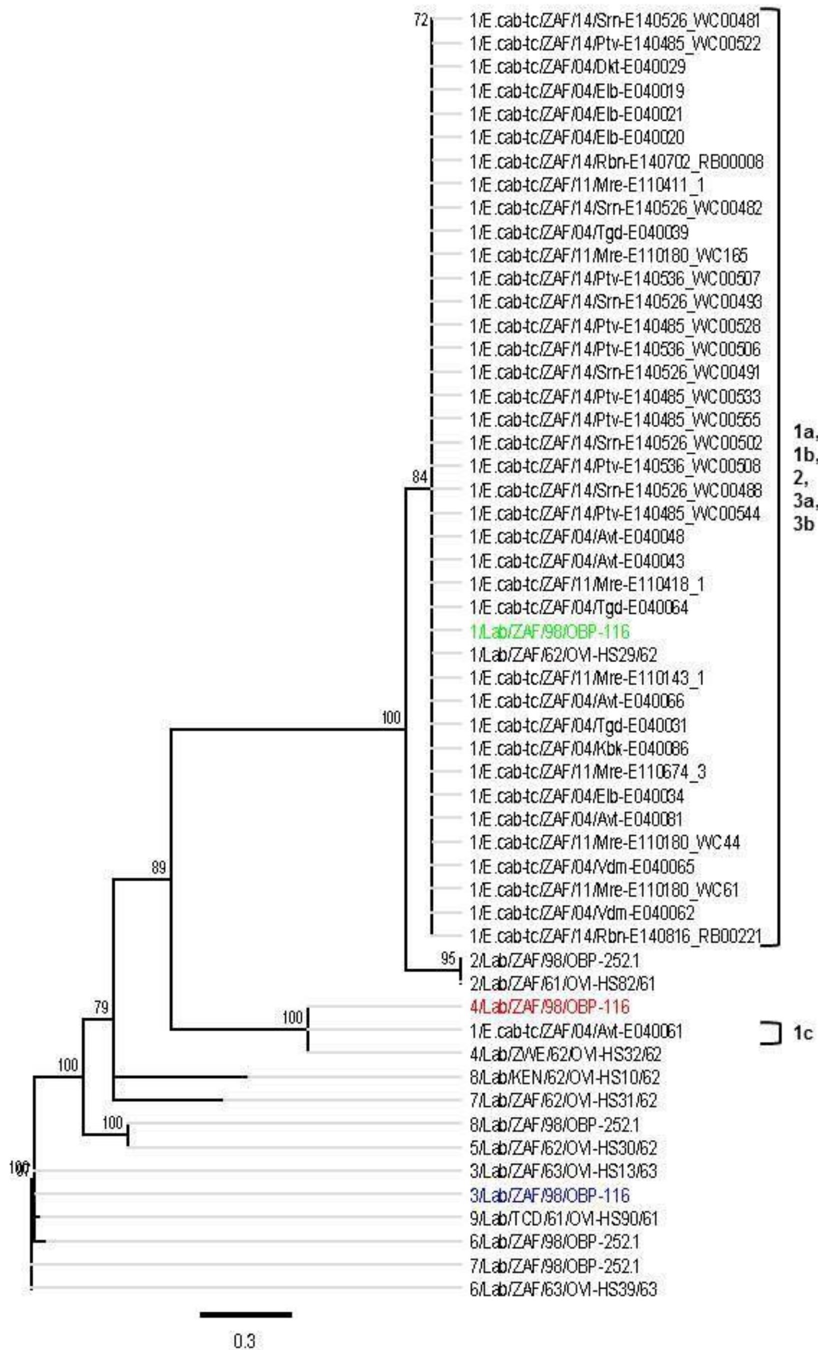


**Figure 6-7: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP5 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details.**



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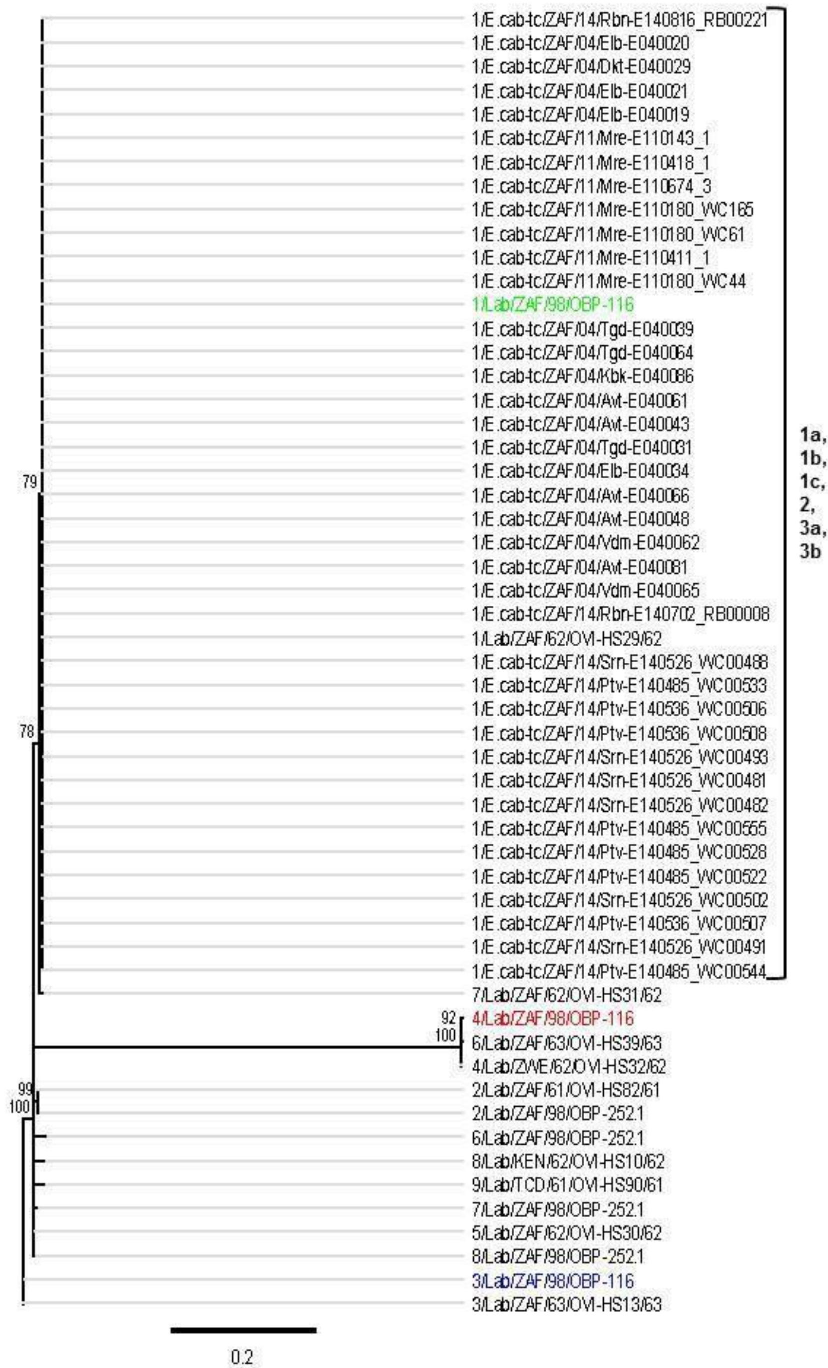


**Figure 6-8: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP6 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details.**



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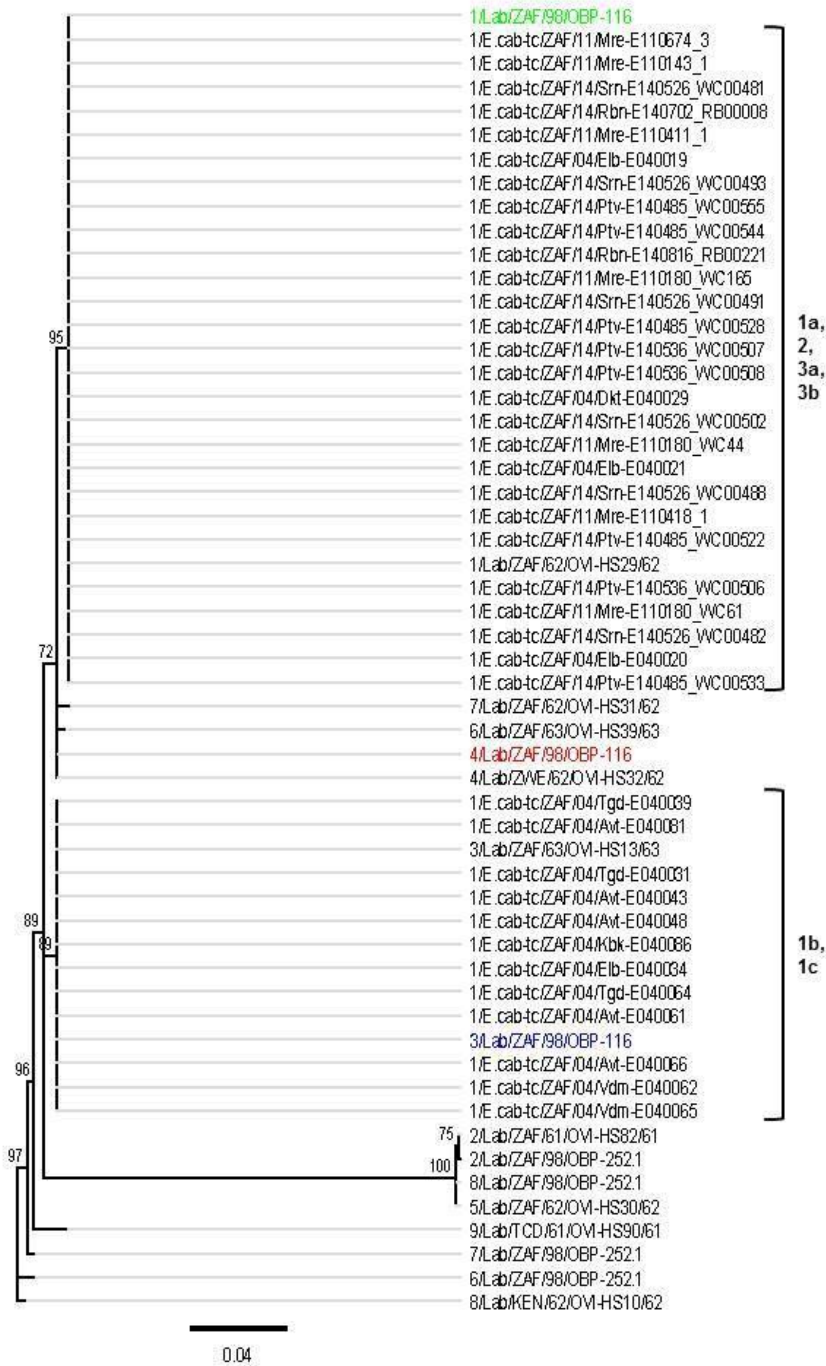


**Figure 6-9: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated VP7 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details.**



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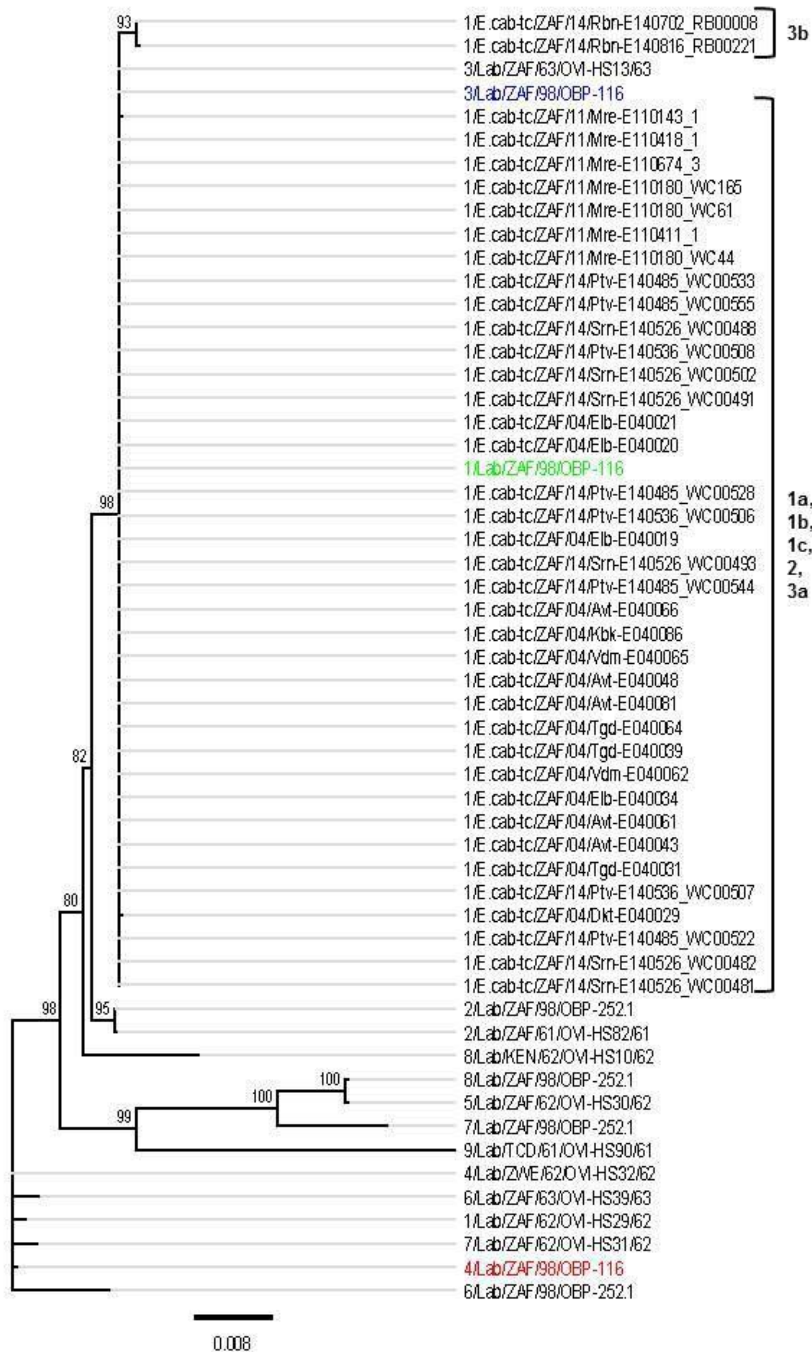


**Figure 6-10: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated NS1 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details.**



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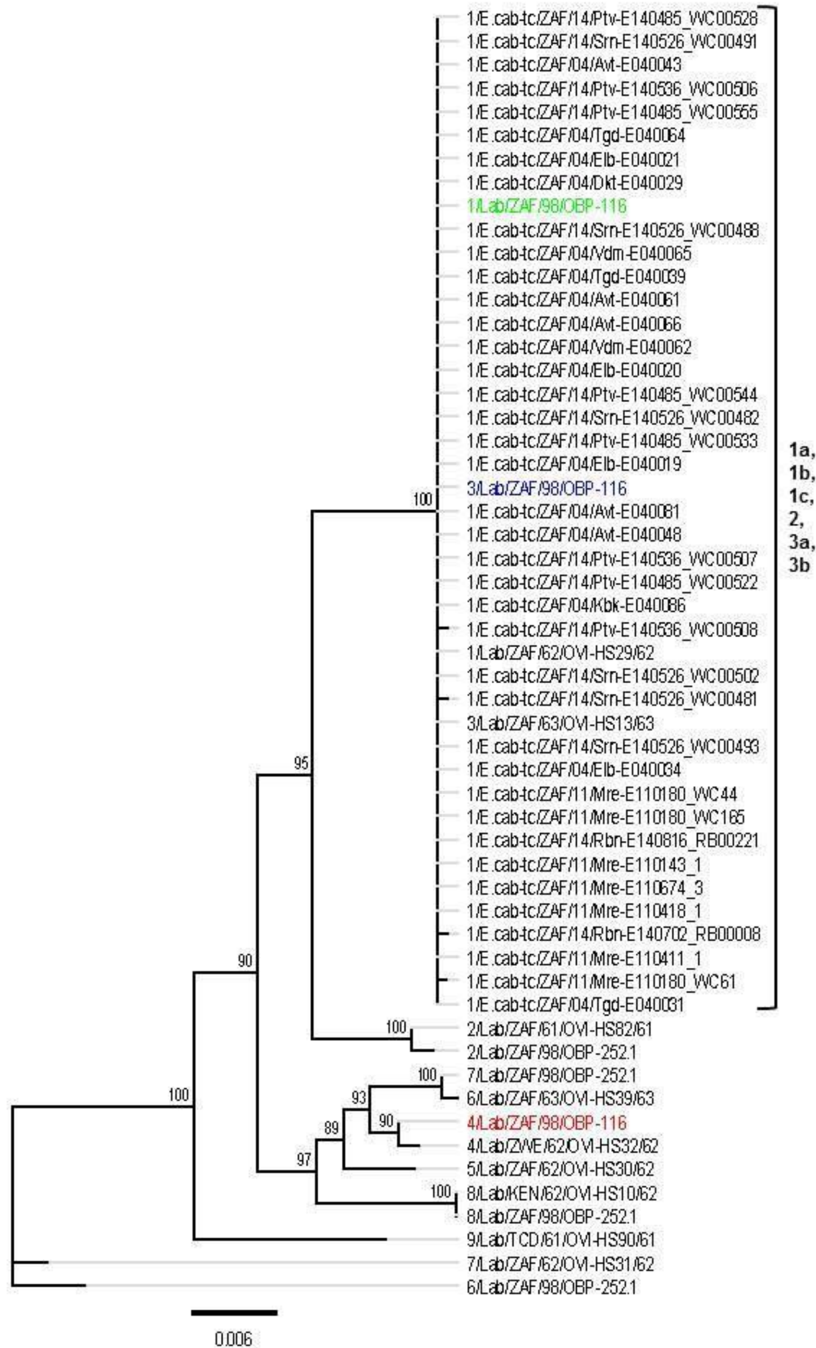


**Figure 6-11: Maximum likelihood phylogenetic tree indicating the genetic relationships of concatenated NS2 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details.**



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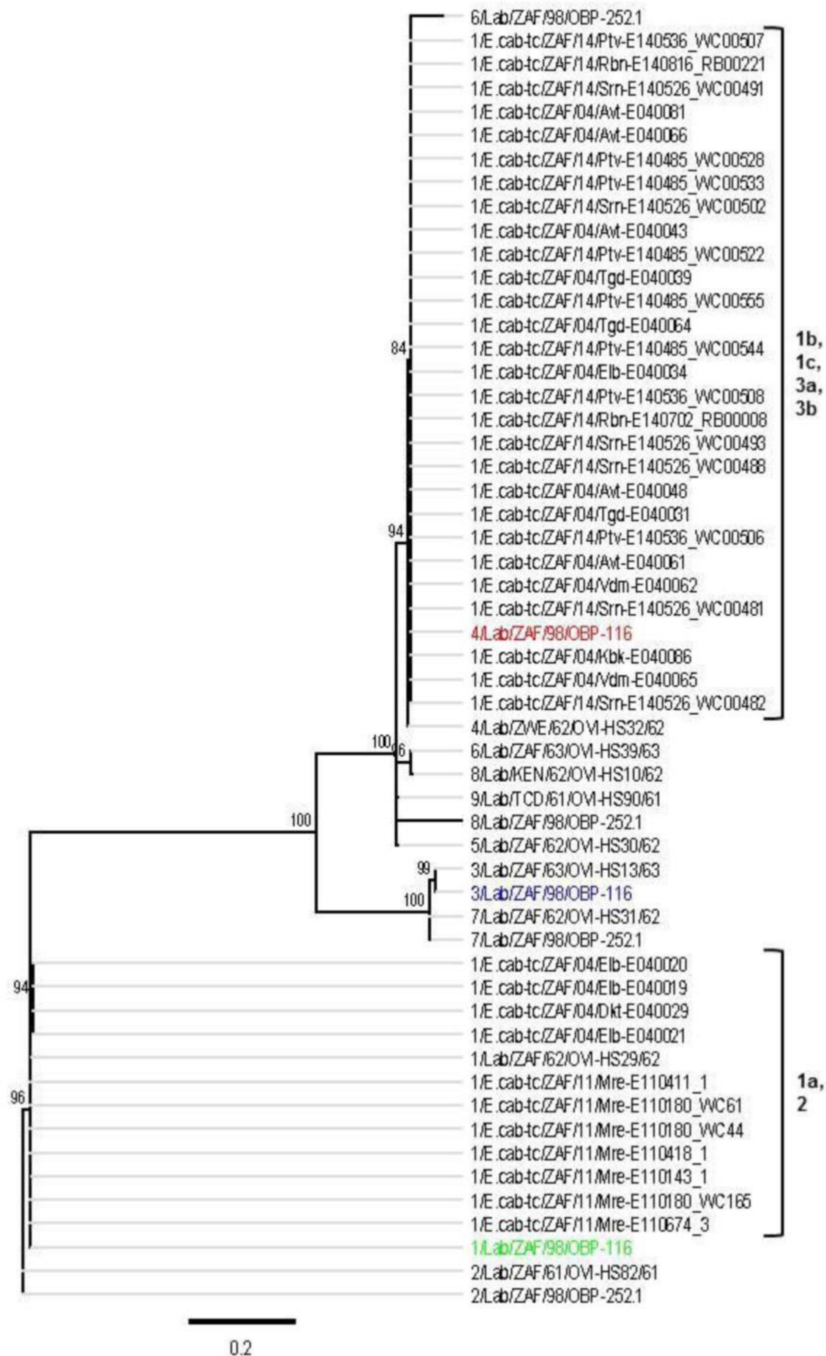


**Figure 6-12: Maximum-likelihood phylogenetic tree indicating the genetic relationships of concatenated NS3 nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS Controlled Area to the AHSV live, attenuated vaccine and reference viruses. See Figure 6-3 for details.**



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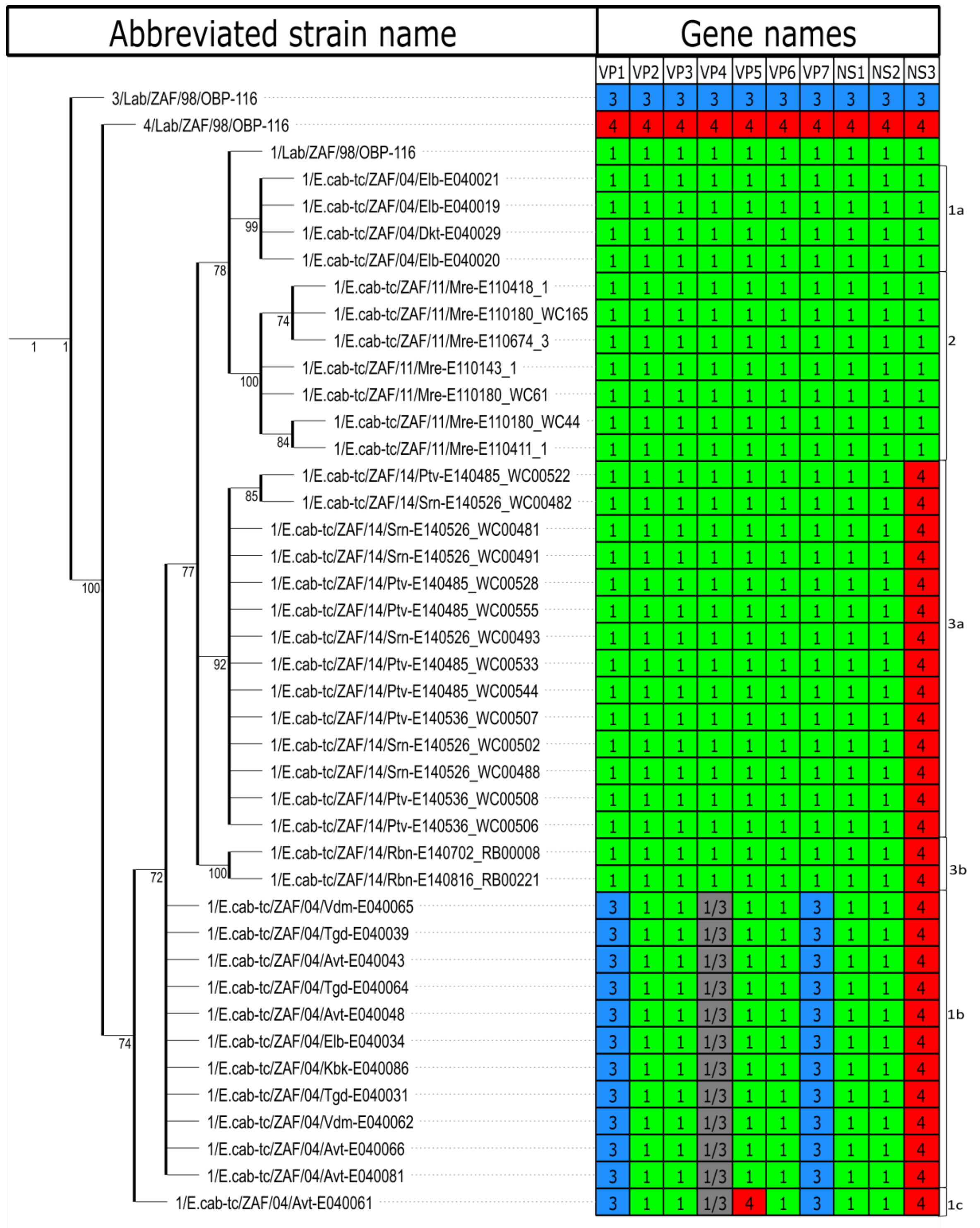


**Figure 6-13: Cladogram and heatmap of vaccine-derived African horse sickness virus reassortants. Cladogram indicating the genetic relationships of concatenated African horse sickness viruses (AHSV) whole genome nucleotide sequences from affected horses in the 2004, 2011 and 2014 outbreaks in the AHS controlled area with a heatmap summarising the origin of the gene segments for each strain with 1/Lab/ZAF/98/OBP-116, 3/Lab/ZAF/98/OBP-116 and 4/Lab/ZAF/98/OBP-116 vaccine derived strains represented by green, blue and red blocks, respectively. Grey blocks indicate that segment could be derived from either 1/Lab/ZAF/98/OBP-116 or 3/Lab/ZAF/98/OBP-116. Branches supported by full maximum likelihood bootstrap values >70% are indicated. Genotype groups are indicated with brackets and group names to the right of the tree.**



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Explicitly testing for statistical evidence of reassortment using eight different recombination/reassortment detection methods in Recombination Detection Program v.4.63 (RDP4) *(106)* strongly supported the occurrence of reassortment in viruses within genotype groups 1b, 1c, 3a and 3b (Table 6-3). While this analysis failed to detect any evidence of intra-component homologous recombination, it confirmed that the viruses included in: 1) genotype group 1a and 2 include viruses with genomes where all ten segments are derived from a virus very closely resembling 1/Lab/ZAF/98/OBP-116, 2); genotype group 1b contains reassortant viruses with six genome segments (encoding VP2, VP3, VP5, VP6, NS1, and NS2) derived from a virus resembling 1/Lab/ZAF/98/OBP-116, two segments (encoding VP1, and VP7) derived from a virus resembling 3/Lab/ZAF/98/OBP-116 (multiple testing corrected P = $2.27 \times 10^{-12}$ and $1.13 \times 10^{-31}$, respectively), a single segment (encoding NS3) derived from a virus resembling 4/Lab/ZAF/98/OBP-116 (P = $9.31 \times 10^{-240}$) and a single segment (encoding VP4) that, due to high degrees of similarity to both 3/Lab/ZAF/98/OBP-116 and 1/Lab/ZAF/98/OBP-116 in this segment (Figure 6-7), could plausibly have been derived from either of these two parental viruses (P = $7.66 \times 10^{-4}$); 3) genotype group 1c includes a reassortant pattern resembling that of group 1b viruses in that its genome has five segments (VP2, VP3, VP6, NS1, NS2) derived from a virus resembling 1/Lab/ZAF/98/OBP-116, two (VP1, VP7) derived from a virus resembling 3/Lab/ZAF/98/OBP-116 (P = $2.27 \times 10^{-12}$ and $6.68 \times 10^{-30}$, respectively) a VP4 sequence from either 1/Lab/ZAF/98/OBP-116 or 3/Lab/ZAF/98/OBP-116 (P = $1.08 \times 10^{-3}$ and two (VP5, NS3) derived from a virus resembling 4/Lab/ZAF/98/OBP-116 (P = $2.95 \times 10^{-298}$ and $1.96 \times 10^{-216}$, respectively); and 4) genotype groups 3a and 3b include reassortant viruses with genomes of nine segments derived from a virus resembling 1/Lab/ZAF/98/OBP-116 and a single segment (NS3) derived from a virus resembling 4/Lab/ZAF/98/OBP-116 (P = $9.31 \times 10^{-240}$) (Figure 6-14).

**Table 6-3: Summary statistics (P values) of tests for reassortment of gene segments of AHSV using seven methods incorporated within RDP4.**

| Method | Gene | | | | |
|---|---|---|---|---|---|
| | VP1 | VP4 | VP5 | VP7 | NS3 |
| **RDP** | NS | NS | $3.60 \times 10^{-298}$ | $1.22 \times 10^{-31}$ | $6.17 \times 10^{-238}$ |
| **GENECONV** | NS | $5.83 \times 10^{-2}$ | $1.87 \times 10^{-293}$ | $1.13 \times 10^{-31}$ | $9.31 \times 10^{-240}$ |
| **Bootscan** | NS | $7.66 \times 10^{-4}$ | $2.95 \times 10^{-298}$ | $1.22 \times 10^{-31}$ | $5.55 \times 10^{-238}$ |
| **Maxchi** | NS | NS | $3.55 \times 10^{-46}$ | $1.39 \times 10^{-5}$ | $1.25 \times 10^{-37}$ |
| **Chimaera** | NS | NS | $8.97 \times 10^{-48}$ | $5.93 \times 10^{-5}$ | $9.88 \times 10^{-38}$ |
| **SiSscan** | NS | NS | $1.29 \times 10^{-63}$ | $6.38 \times 10^{-6}$ | $2.30 \times 10^{-45}$ |
| **3Seq** | $2. 27 \times 10^{-12}$ | NS | $1.22 \times 10^{-13}$ | NS | $2.20 \times 10^{-182}$ |



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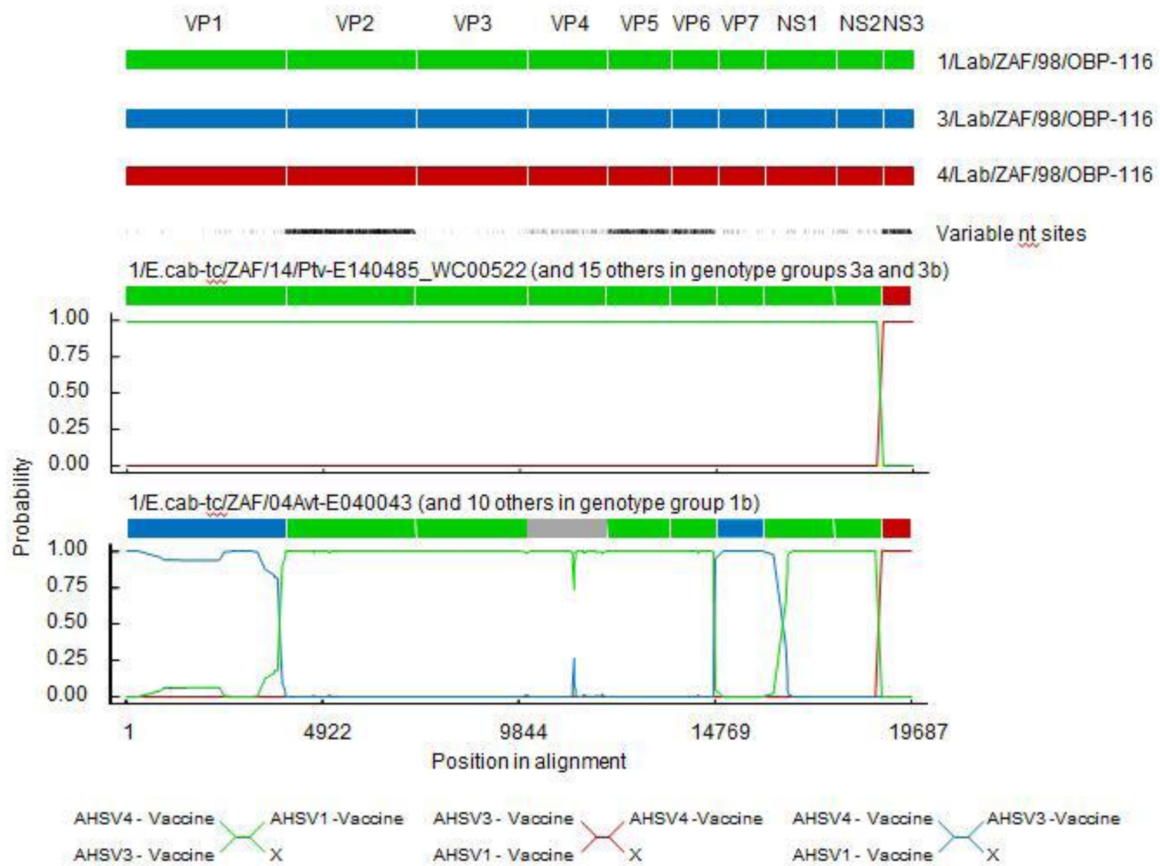


**Figure 6-14: Statistical evidence of reassortment within the genomes of AHSV field isolates. A Hidden Markov model based approach (called BURT-HMM) was used to classify individual nucleotides within each of the 10 segments of individual AHSV isolates into three different categories: 1/Lab/ZAF/98/OBP-116-like (in green), 3/Lab/ZAF/98/OBP-116-like (in blue) and 4/Lab/ZAF/98/OBP-116-like (in red). Probability supports for these classifications yielded by the BURT-HMM with the highest likelihood are plotted along the genome with the positions of segment boundaries given in the diagram above the plots. The phylogenetic clusterings that are implied by differently coloured segments in these plots are indicated below the plots. Note that the segment indicated in grey could not be convincingly classified because it very closely resembles both 1/Lab/ZAF/98/OBP-116 and 3/Lab/ZAF/98/OBP-116.**

A number of interesting single nucleotide variations (SNVs) relative to the AHSV-LAV derived viruses 1/Lab/ZAF/98/OBP-116 and 3/Lab/ZAF/98/OBP-116 are present in the NS1 encoding genes of viruses included in genotype groups 3a (2014 Porterville) and 3b (2014 Robertson; Table 6-4). There is only a single non-synonymous SNV (nsSNV) between the NS1-encoding genes of 1/Lab/ZAF/98/OBP-116 and 3/Lab/ZAF/98/OBP-116 (NS1 I264T). All viruses included in genotype group 3a (2014 Porterville) have the I amino acid variant that is present in 1/Lab/ZAF/98/OBP-116, whereas viruses in genotype group 3b (2014 Robertson) include the T amino acid variant present in 3/Lab/ZAF/98/OBP-116. Viruses in the 3b genotype group (2014 Robertson) include at least two synonymous SNVs and at least one nsSNV relative to 3/Lab/ZAF/98/OBP-116, which suggests that the NS1 gene of the virus strains in genotype group 3b are most probably derived from 3/Lab/ZAF/98/OBP-116



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whereas those in genotype group 3a are more likely to be derived from 1/Lab/ZAF/98/OBP-116.

**Table 6-4: Summary of single nucleotide variations (SNVs) observed in NS1 of 1/Lab/ZAF/98/OBP-116 and 3/Lab/ZAF/98/OBP-116 vaccine derived strains and strains isolated during 2014 AHS outbreaks in Porterville and Robertson. [a] Position given as nucleotide and (amino acid). [b] SNV's given as nucleotide and (amino acid).**

| Position | Virus Strains | | | | |
|---|---|---|---|---|---|
| | 1/OBP-116 (+11) | 3/OBP-116 | 1/Srn-WC00481 | 1/RB00008 | 1/RB00221 |
| 839 (268)[a] | U (A)[b] | U (A) | U (A) | A (A) | A (A) |
| 884 (283) | U (Y) | U (Y) | C (Y) | U (Y) | U (Y) |
| 1126 (264) | U (I) | C (T) | U (I) | C (T) | C (T) |
| 1191 (386) | U (L) | U (L) | U (L) | C (L) | C (L) |
| 1382 (449) | G (T) | G (T) | G (T) | G (T) | A (T) |
| 1392 (453) | G (E) | G (E) | G (E) | C (Q) | C (Q) |

A total of seven nsSNVs were identified between the whole genome sequences of the AHSV-1-LAV derived virus, 1/Lab/ZAF/98/OBP-116, and its parental virus, 1/E.cab-tc/ZAF/62/OVI-HS29/62 (Table 6-5). SNVs are present at four of these seven sites in the four viruses included in genotype group 1a. Intriguingly, three of these four changes are apparently reversions to the nsSNV that is present in the virulent parental virus (I434T in VP5 and V81A and Q169R in VP6) and are therefore potentially reversion-to-virulence mutations. The one other SNV in the genotype group 1a viruses is site 201 in NS3: whereas in 1/Lab/ZAF/98/OBP-116 and 1/E.cab-tc/ZAF/62/OVI-HS29/62 there is a K and an M, respectively, at this site, in the group 1a viruses there is an E at this site. In ten of the eleven field viruses in genotype group 1b, nsSNVs were also detected at four of the seven sites that differentiate the attenuated 1/Lab/ZAF/98/OBP-116 virus from its virulent parent, 1/E.cab-tc/ZAF/62/OVI-HS29/62. The remaining field virus in genotype group 1b, 1/E.cab-tc/ZAF/04/Vdm-E040065, includes three of these four SNVs. The I434T SNV in VP5, and the V81A and Q169R SNVs in VP6 of viruses in genotype group 1a are the same as those found in the genotype group 1b, 2, 3a and 3b viruses. The K357N SNV in VP2 was detected only amongst viruses in genotype groups 1b and 1c. All the viruses included in genotype groups 1b, 1c, 3a and 3b are also reassortants with a NS3 encoding segment derived from a virus resembling 4/Lab/ZAF/98/OBP-116 and so the SNVs in this component of these viruses were not considered as genuine mutationally derived SNVs.



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**Table 6-5: A summary of the attenuation-associated non-synonymous single nucleotide variants (nsSNVs) of the consensus sequences of the genome segments of the African horse sickness type 1 (AHSV-1) Laboratory Reference Strain, 1/Lab/ZAF/98/OBP-116 vaccine-derived virus and AHSV-1 viruses from the 2004 Stellenbosch, 2011 Mamre, 2014 Porterville and 2014 Robertson AHS outbreaks.**

| Abbreviated Strain Name | Genome Segment and Amino Acid Position | | | | | | | Genotype group |
|---|---|---|---|---|---|---|---|---|
| | VP2 | VP3 | VP5 | | VP6 | | NS3 | |
| | 357 | 232 | 422 | 434 | 81 | 169 | 201 | |
| 1/E.cab-tc/ZAF/62/OVI-HS29/62 | N | Y | S | T | A | R | M | |
| 1/Lab/ZAF/98/OBP-116[a] | K | H | N | I | V | Q | K | |
| 1/E.cab-tc/ZAF/04/Elb-E040019 | [b] | | | T[c] | A | R | E | 1a |
| 1/E.cab-tc/ZAF/04/Elb-E040020 | | | | T | A | R | E | 1a |
| 1/E.cab-tc/ZAF/04/Elb-E040021 | | | | T | A | R | E | 1a |
| 1/E.cab-tc/ZAF/04/Dkt-E040029 | | | | T | A | R | E | 1a |
| 1/E.cab-tc/ZAF/04/Tgd-E040031 | N | | | T | A | R | -[d] | 1b |
| 1/E.cab-tc/ZAF/04/Elb-E040034 | N | | | T | A | R | - | 1b |
| 1/E.cab-tc/ZAF/04/Tgd-E040039 | N | | | T | A | R | - | 1b |
| 1/E.cab-tc/ZAF/04/Avt-E040043 | N | | | T | A | R | - | 1b |
| 1/E.cab-tc/ZAF/04/Avt-E040048 | N | | | T | A | R | - | 1b |
| 1/E.cab-tc/ZAF/04/Vdm-E040062 | N | | | T | A | R | - | 1b |
| 1/E.cab-tc/ZAF/04/Tgd-E040064 | N | | | T | A | R | - | 1b |
| 1/E.cab-tc/ZAF/04/Vdm-E040065 | N | | | T | A | | - | 1b |
| 1/E.cab-tc/ZAF/04/Avt-E040066 | N | | | T | A | R | - | 1b |
| 1/E.cab-tc/ZAF/04/Avt-E040081 | N | | | T | A | R | - | 1b |
| 1/E.cab-tc/ZAF/04/Kbk-E040086 | N | | | T | A | R | - | 1b |
| 1/E.cab-tc/ZAF/04/Avt-E040061 | N | | - | - | A | R | - | 1c |
| 1/E.cab-tc/ZAF/11/Mre-E110143_1 | | | | T | A | R | N | 2 |
| 1/E.cab-tc/ZAF/11/Mre-E110180_WC44 | | | | T | A | R | N | 2 |
| 1/E.cab-tc/ZAF/11/Mre-E110180_WC61 | | | | T | A | R | N | 2 |
| 1/E.cab-tc/ZAF/11/Mre-E110180_WC165 | | | | T | A | R | N | 2 |
| 1/E.cab-tc/ZAF/11/Mre-E110411_1 | | | | T | A | R | N | 2 |
| 1/E.cab-tc/ZAF/11/Mre-E110418_1 | | | | T | A | R | N | 2 |
| 1/E.cab-tc/ZAF/11/Mre-E110674_3 | | | | T | A | R | N | 2 |
| 1/E.cab-tc/ZAF/14/Ptv-E140485_WC00522 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Ptv-E140485_WC00528 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Ptv-E140485_WC00533 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Ptv-E140485_WC00544 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Ptv-E140485_WC00555 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Srn-E140526_WC00481 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Srn-E140526_WC00482 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Srn-E140526_WC00488 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Srn-E140526_WC00491 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Srn-E140526_WC00493 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Srn-E140526_WC00502 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Ptv-E140536_WC00506 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Ptv-E140536_WC00507 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Ptv-E140536_WC00508 | | | | T | A | R | - | 3a |
| 1/E.cab-tc/ZAF/14/Rbn-E140702_RB00008 | | | | T | A | R | - | 3b |
| 1/E.cab-tc/ZAF/14/Rbn-E140816_RB00221 | | | | T | A | R | - | 3b |

a The changes in amino acids are indicated as compared to the AHSV-1-LAV derived strain (1/Lab/ZAF/98/OBP-116) for relevant viral proteins.

b Sequences that were identical to the consensus sequence of the vaccine derived strain are indicated by an empty block.

c Sequences that differed from the consensus sequence of the AHSV-1-LAV derived strain are indicated with the letter symbol of the relevant amino acid.

d Indicates that these segments were not considered due to a recombination event that occurred with another vaccine-derived AHSV type.



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The 1/E.cab-tc/ZAF/04/Avt-E040061 strain in genotype group 1c has nsSNVs at three of the seven loci (K357N in VP2 and V81A and Q169R in VP6), but a VP5 encoding segment apparently derived by reassortment from a virus resembling 4/Lab/ZAF/98/OBP-116 such that the SNVs in the VP5 of this strain was also not considered to be a mutationally derived.

The seven viruses included in genotype group 2 and the 16 viruses included in genotype groups 3a and 3b all exhibit potential reversion-to-virulence mutations at three of the seven nsSNV sites that differentiate the AHSV-1-LAV virus from its virulent parent (I434T in VP5 and V81A and Q169R in VP6). Additionally, a fourth SNV (K201N in NS3) at one of the seven sites differentiating the AHSV-1-LAV from its parent (which had a K and an M, respectively, at this site) is also present in the genotype group 2 viruses.

***Quantification of the outbreaks***

The epidemiological parameters of the AHS outbreaks in the CA in 2004, 2011 and 2014, were inferred using the current OIE case definition for AHS (Table 6-6) *(180)*. Whereas the case fatality rates (CFRs) were very high for the 2004 Stellenbosch and 2011 Mamre outbreaks (78.3% and 76.2%, respectively), they were considerably lower for the 2014 Porterville (14.6%) and Robertson (4.5%) outbreaks. However, there were other differences between these outbreaks; for example, the 2011 Mamre and 2004 Stellenbosch outbreaks were associated with the lowest case vaccination statuses (2.7% and 8.7%, respectively). Clearly, differences in the genetic constitution of the individual outbreak viruses could have been associated with the vastly different CFRs in each outbreak, and the viruses included in genotype groups 3a (Porterville) and 3b (Robertson) were associated with especially low CFRs, which is probably indicative of a high rate of subclinical AHSV infection of horses during the 2014 outbreaks. However, it is currently unknown if these differences in CFRs between outbreaks are a consequence of lower virulence amongst the outbreak viruses or whether they are attributable to existing vaccine-induced immunity in the exposed horses. Similarly, changes in the AHS case definition that only came into effect in 2008 (after the 2004 Stellenbosch outbreak) probably resulted in an underestimation of subclinical AHSV infections during that outbreak. Specifically, whereas during the Stellenbosch 2004 outbreak only clinically affected, deceased horses were classified as confirmed cases *(155)*, there have been major advances in AHS diagnostic testing over the past 10 years (such as RT-qPCR based methods) that likely substantially increased the detectability of subclinical infections by the time of the 2014 outbreak *(56, 176)*.



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**Table 6-6: Summary of the epidemiological parameters for each of the outbreaks involving AHSV-1 in the AHS controlled area of the Western Cape Province of South Africa in 2004, 2011 and 2014.**

| Parameter | 2004 Stellenbosch | 2011 Mamre | 2014 Porterville | 2014 Robertson |
|---|---|---|---|---|
| **Confirmed cases** | 23 (16)[a] | 84 (73)[b] | 89 | 22 |
| **Deaths** | 18 (16)[a] | 64 (64)[b] | 13 | 1 |
| **Case fatality rate** | 78.3% (100%)[a] | 76.2% (87.7%)[b] | 14.6% | 4.5% |
| **Subclinical cases** | 0 | 15 (4)[b] | 52 | 17 |
| **% subclinical** | 0 | 17.9% (5.5%)[b] | 58.4% | 77.3% |
| **Vaccinated cases** | 2/23 | 2/84 | 35/89 | 3/22 |
| **% vaccinated** | 8.7% | 2.4% | 39.3% | 13.6% |
| **Properties affected** | 10 (8)[a] | 47 (45)[b] | 31 | 8 |

The parameters are calculated using the current OIE case definition and parameters calculated using the case definitions when the outbreaks occurred are in parenthesis for the 2004 and 2011 outbreaks.

a An additional 5 clinical cases and 2 deaths that met the criteria of the current OIE AHS case definition were not included using the case definition in place at the time of this outbreak *(154)*.

b An additional 11 subclinical cases that met the criteria of the current OIE AHS case definition were not included using the case definition in place at the time of this outbreak *(56)*.

## 6.5 Discussion

A total of 55 field, AHSV-LAV, and laboratory strains of AHSV were subjected to whole genome sequencing and detailed phylogenetic analyses. The field viruses were obtained from horses during outbreaks of AHS of remarkably different clinical severity (CFRs ranging from 4.5 – 78.3%) in the AHS CA of the Western Province during 2004, 2011, and 2014. Deep sequencing and phylogenetic analyses confirmed that genetically distinct viruses were responsible for each outbreak, and that these were all closely related to viruses contained in the trivalent (OBP comb1, AHSV-1, 3 and 4) AHSV-LAV used in South Africa. Evaluation of nsSNVs confirmed some outbreak viruses to be revertants of the vaccine AHSV-1 strain towards the virulent parental type. Furthermore, some outbreak viruses were clearly reassortants with individual genome segments derived from multiple different virus types that are present in the trivalent vaccine preparation.

Potgieter and co-workers *(132)* hypothesized that changes in both VP2 and VP5 can confer virulence and/or attenuation of individual AHSV strains, based upon comparisons of the consensus sequences of the genome of an attenuated strain of AHSV1 (FJ183364-FJ183373) and its virulent parent. It is proposed that virulence is related to tissue tropism as the outer capsid proteins are involved in cell entry and trigger the apoptosis of host cells *(44, 132, 132)*. Additionally, others have implicated NS3 as a determinant of AHSV virulence *(107, 123)*. The results of our study further confirm that changes in multiple viral proteins can



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affect the virulence of AHSV, in that both reversion (to the virulent parental type) and novel SNVs were present in field-isolated viruses at sites in VP2 (K357N in genotype group 1b viruses), VP5 (I434T in all field viruses evaluated except sample 1/E.cab-tc/ZAF/04/Avt-E040061) and VP6 (V81A in all field viruses, and Q169R in all field viruses except sample 1/E.cab-tc/ZAF/04/Vdm-E040065) that differentiates the attenuated AHSV-1-LAV strain from its virulent parent strain. Furthermore SNVs present at a site in NS3 (K201E in genotype group 1a viruses and K201N in genotype group 2) are potentially associated with reversion to virulence due to the proteins effect on virus release, membrane permeability and total viral yield *(111)*. It is probable, however, that the determinants of AHSV virulence are complex and multigenic *(84, 132)*, which is consistent with the remarkable difference in CFRs between horses in the various outbreaks despite the relatively similar genetic constitution of the viruses isolated from these horses and the close relationships of these viruses to the AHSV-LAVs from which they were likely derived.

Given the genetic diversity of field strains of AHSV *(131, 132, 138)*; our analyses overwhelmingly support the premise that the potential reversion-to-virulence mutants and reassortants that we detected arose from viruses within the polyvalent AHSV-LAV formulation, and predominantly from AHSV-1-LAV. While it is most likely that these mutants and reassortants arose within vaccinated horses, it is currently uncertain as to why components of AHSV-1-LAV predominate in the emergent outbreak viruses. Interestingly, the data presented here also indicate that distinct founder events lead to the expansion in Stellenbosch (2004) of viruses included in genotype groups 1a and 1b and, similarly, that the outbreaks in 2014 in Porterville (genotype group 3a) and Robertson (genotype group 3b) also likely originated independently from the LAV and not from the spread of the same outbreak virus.

Reassortment is well established for bluetongue virus (BTV) *(125, 148, 149, 160)*, the prototype virus of the genus *Orbivirus*, and multiple reassortment events between field and LAV strains of BTV were documented during recent epidemics in Europe *(121)*. Natural transmission of AHSV-9-LAV has also been described recently in the Gambia *(126)*. Given the recent emergence of several AHSV types in the northern margins of sub-Saharan Africa *(2, 184)*, these outbreak viruses should be compared to any AHSV-LAVs used in the region. However, any such analyses would further require the availability of sequence data from these vaccines, as recently proposed for other LAVs *(28)*.

In summary, the in-depth genetic analyses described here highlight the importance in epidemiological investigations of carefully characterizing field strains of AHSV so as to



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accurately determine the origin of AHS outbreaks. Whereas existing dogma in South Africa was that illegal movements of viraemic equids into the AHS CA of this country were responsible for the repeated occurrences of AHS in the CA, this is clearly not the only cause. The results we report here indicate that use of polyvalent AHSV-LAV can result in the emergence and spread of virulent viruses to adjacent susceptible horses –presumably by *Culicoides* midge vectors which are known to be present within the AHS CA of the Western Cape Province *(119, 168)*. Collectively, these findings have significant implications for strategies to control AHS, both in endemic regions and during future incursions of AHSV into currently virus-free areas. However, it must be emphasized that the AHSV-LAV vaccine provides critical and effective protection of susceptible horses in endemic areas and, although potentially safer recombinant AHSV vaccines have proven effective in laboratory studies, these are not available commercially and they are yet to be evaluated in the field *(3, 63)*. Until such time as alternative vaccines become commercially available, therefore, control of AHS will remain reliant on the use of the AHSV-LAV vaccine coupled, ideally, with the adoption of strategies to minimize the likelihood of natural dissemination of revertant and/or reassortant vaccine-derived viruses.



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# GENERAL CONCLUSIONS

Chapter 2 gives a detailed description of the AHS outbreak that occurred in the surveillance zone of the AHS controlled area in 2011. It highlights the need for accurate and fast diagnostic techniques, especially in the form of AHS typing. Furthermore the confounding effect of subclinical cases, as well as that of vaccination in the face of an outbreak, on the surveillance strategies that can be implemented is highlighted.

Chapter 3 gives a solution to the need for accurate, fast AHSV typing. This chapter describes three triplex, AHSV type specific RT-qPCR assays for the rapid identification of the AHSV type involved in group specific RT-qPCR positive samples. The chapter describes the use in field conditions, as well identifying some issues with the historical and current AHSV reference strains.

Chapter 4 evaluates the use of foals as a sentinel surveillance population, especially following an outbreak. The difficulty in interpretation of maternal antibody in foals was shown, and an alternative in the form of RT-qPCR, or better yet as a supplementary test to be used in conjunction with serology, was shown.

Chapter 5 highlighted the fact that horses can be positive on RT-qPCR for extended periods of time, and this is due to multiple AHSV types. This has obvious implications in AHS surveillance, but also suggests that AHSV viraemia is likely to occur, and therefore vaccine transmission possible under favourable circumstances.

The dissertation culminates in the molecular and epidemiological comparison of the five unique AHSV type 1 outbreaks in the AHS controlled area since 1997. Chapter 6 describes the occurrence of reversion to virulence of the strains of AHSV contained in OBP comb1 of the modified AHSV-LAV to the virulent parental strain leading to outbreaks of AHSV in the AHS CA. Furthermore it describes a number of reassortment events that have occurred and the subsequent transmission and resulting outbreaks that have occurred within the CA.

The findings described in Chapter 5 together with the recent documentation that many of the AHS outbreaks in the Western Cape AHS controlled area were caused by transmission of vaccine derived viruses (Chapter 6) shows the importance of responsible use of an AHSV-LAV, especially in an area of low prevalence where imprudent use of such a vaccine could in fact cause outbreaks of AHS.



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The chapters in this thesis collectively show the importance of molecular testing in AHS outbreak investigation and disease surveillance. The RNAaemia in horses following vaccination with the commercial AHS ALV vaccine has been described and the importance shown in the molecular investigations of the outbreaks in the AHS controlled area. The outbreak descriptions and AHS foal surveillance strategy provides a firm scientific basis for decision making and further outbreak response and surveillance strategy development which are fundamental to support export protocols for horses from South Africa.



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# APPENDICES

## APPENDIX A

**Results of virus isolation (VI), plaque inhibition (PI), group specific (AHSV RT-qPCR) and type specific (AHSV TS RT-qPCR) real time PCR's for all samples included in the study.**

| Laboratory Reference | VI | PI | AHSV RT-qPCR Cq | AHSV TS RT-qPCR Cq | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| **E143/11_02** | Negative | * | 26.8 | 22.0 | * | * | * | * | * | * | * | * |
| **E412/11_02** | Negative | * | 21.0 | 22.4 | * | * | * | * | * | * | * | * |
| **E480/11** | Negative | * | 21.9 | 22.9 | * | * | * | * | * | * | * | * |
| **E507/11** | Negative | * | 27.7 | 25.7 | * | * | * | * | * | * | * | * |
| **E512/11** | Negative | * | 25.4 | 24.3 | * | * | * | * | * | * | * | * |
| **E546/11** | Negative | * | 30.6 | 20.4 | * | * | * | * | * | * | * | * |
| **E564/11** | Negative | * | 19.4 | 20.4 | * | * | * | * | * | * | * | * |
| **E587/11_02** | Negative | * | 26.3 | 26.9 | * | * | * | * | * | * | * | * |
| **E587/11_03** | Negative | * | 26.2 | 27.0 | * | * | * | * | * | * | * | * |
| **E621/11** | Negative | * | 28.4 | 30.5 | * | * | * | * | * | * | * | * |
| **E660/11** | Negative | * | 26.7 | 27.7 | * | * | * | * | * | * | * | * |
| **E664/11** | Negative | * | 30.0 | 29.2 | * | * | * | * | * | * | * | * |
| **E699/11** | Negative | * | 26.5 | 24.6 | * | * | * | * | * | * | * | * |
| **WC013** | Negative | * | 28.1 | 28.6 | * | * | * | * | * | * | * | * |
| **WC015/11** | Negative | * | 28.2 | 28.6 | * | * | * | * | * | * | * | * |
| **WC037** | Negative | * | 28.8 | 29.4 | * | * | * | * | * | * | * | * |
| **WC038** | Negative | * | 26.2 | 26.4 | * | * | * | * | * | * | * | * |
| **WC054** | Negative | * | 22.9 | 23.3 | * | * | * | * | * | * | * | * |
| **WC056** | Negative | * | 28.6 | 28.7 | * | * | * | * | * | * | * | * |
| **WC081** | Negative | * | 29.1 | 29.2 | * | * | * | * | * | * | * | * |
| **WC161** | Negative | * | 27.1 | 27.7 | * | * | * | * | * | * | * | * |
| **WC163** | Negative | * | 29.7 | 30.4 | * | * | * | * | * | * | * | * |
| **WC164** | Negative | * | 22.6 | 22.7 | * | * | * | * | * | * | * | * |
| **E049/11_02** | Negative | * | 27.3 | * | 30.3 | * | * | * | * | * | * | * |
| **E067/11** | Negative | * | 29.3 | * | 28.2 | * | * | * | * | * | * | * |
| **E070/11** | Negative | * | 25.9 | * | 29.5 | * | * | * | * | * | * | * |
| **E073/11_04** | Negative | * | 29.0 | * | 33.1 | * | * | * | * | * | * | * |
| **E140/12** | Negative | * | 28.7 | * | 33.9 | * | * | * | * | * | * | * |
| **E157/11** | Negative | * | 25.6 | * | 28.1 | * | * | * | * | * | * | * |
| **E162/12** | Negative | * | 25.6 | * | 30.7 | * | * | * | * | * | * | * |
| **E193/11** | Negative | * | 25.8 | * | 31.9 | * | * | * | * | * | * | * |
| **E215/11** | Negative | * | 29.9 | * | 29.5 | * | * | * | * | * | * | * |
| **E261/11_01** | Negative | * | 23.0 | * | 27.8 | * | * | * | * | * | * | * |
| **E317/11** | Negative | * | 24.8 | * | 28.7 | * | * | * | * | * | * | * |
| **E376/11_01** | Negative | * | 25.5 | * | 28.9 | * | * | * | * | * | * | * |
| **E376/11_02** | Negative | * | 25.7 | * | 30.0 | * | * | * | * | * | * | * |
| **E389/11_01** | Negative | * | 27.6 | * | 31.6 | * | * | * | * | * | * | * |
| **E400/11** | Negative | * | 27.3 | * | 31.5 | * | * | * | * | * | * | * |
| **E422/11_01** | Negative | * | 28.8 | * | 31.6 | * | * | * | * | * | * | * |
| **E427/11_01** | Negative | * | 26.5 | * | 28.3 | * | * | * | * | * | * | * |
| **E444/11** | Negative | * | 28.1 | * | 24.9 | * | * | * | * | * | * | * |
| **E470/11** | Negative | * | 29.9 | * | 28.3 | * | * | * | * | * | * | * |
| **E493/11_01** | Negative | * | 23.9 | * | 27.3 | * | * | * | * | * | * | * |
| **E494/11** | Negative | * | 29.0 | * | 32.8 | * | * | * | * | * | * | * |
| **E547/11_01** | Negative | * | 27.1 | * | 31.8 | * | * | * | * | * | * | * |
| **E547/11_02** | Negative | * | 25.3 | * | 29.3 | * | * | * | * | * | * | * |



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| Laboratory Reference | VI | PI | AHSV RT-qPCR Cq | AHSV TS RT-qPCR Cq | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| **E628/11** | Negative | * | 29.7 | * | 30.6 | * | * | * | * | * | * | * |
| **E647/11** | Negative | * | 28.2 | * | 32.8 | * | * | * | * | * | * | * |
| **E706/11** | Negative | * | 26.3 | * | 26.4 | * | * | * | * | * | * | * |
| **E463/11** | Negative | * | 24.2 | * | * | 24.8 | * | * | * | * | * | * |
| **E053/11** | Negative | * | 28.5 | * | * | * | 29.2 | * | * | * | * | * |
| **E168/11** | Negative | * | 28.3 | * | * | * | 34.4 | * | * | * | * | * |
| **E175/11_02** | Negative | * | 28.3 | * | * | * | 33.3 | * | * | * | * | * |
| **E200/11** | Negative | * | 25.2 | * | * | * | 29.6 | * | * | * | * | * |
| **E257/11** | Negative | * | 29.9 | * | * | * | 33.4 | * | * | * | * | * |
| **E403/11** | Negative | * | 23.0 | * | * | * | 28.2 | * | * | * | * | * |
| **E469/11** | Negative | * | 27.8 | * | * | * | 31.1 | * | * | * | * | * |
| **E483/11** | Negative | * | 26.9 | * | * | * | 30.6 | * | * | * | * | * |
| **E635/11** | Negative | * | 26.4 | * | * | * | 28.3 | * | * | * | * | * |
| **E678/11** | Negative | * | 25.1 | * | * | * | 31.8 | * | * | * | * | * |
| **E718/11_01** | Negative | * | 26.9 | * | * | * | 36.5 | * | * | * | * | * |
| **E896/11** | Negative | * | 28.5 | * | * | | 34.0 | * | * | * | * | * |
| **E065/11** | Negative | * | 24.9 | * | * | * | * | 27.1 | * | * | * | * |
| **E139/12** | Negative | * | 26.8 | * | * | * | * | 31.2 | * | * | * | * |
| **E293/11** | Negative | * | 29.3 | * | * | * | * | 33.1 | * | * | * | * |
| **E294/11** | Negative | * | 30.0 | * | * | * | * | 33.9 | * | * | * | * |
| **E296/11** | Negative | * | 27.9 | * | * | * | * | 29.8 | * | * | * | * |
| **E318/11** | Negative | * | 28.2 | * | * | * | * | 33.7 | * | * | * | * |
| **E389/11_02** | Negative | * | 25.4 | * | * | * | * | 27.8 | * | * | * | * |
| **E391/11** | Negative | * | 26.1 | * | * | * | * | 28.6 | * | * | * | * |
| **E474/11_05** | Negative | * | 29.6 | * | * | * | * | 34.7 | * | * | * | * |
| **E493/11_02** | Negative | * | 25.5 | * | * | * | * | 31.3 | * | * | * | * |
| **E561/11_01** | Negative | * | 28.1 | * | * | * | * | 32.5 | * | * | * | * |
| **E668/11** | Negative | * | 27.7 | * | * | * | * | 32.6 | * | * | * | * |
| **E687/11** | Negative | * | 29.4 | * | * | * | * | 34.0 | * | * | * | * |
| **E077/12** | Negative | * | 27.1 | * | * | * | * | * | 29.9 | * | * | * |
| **E148/11** | Negative | * | 29.5 | * | * | * | * | * | 31.3 | * | * | * |
| **E351/11** | Negative | * | 28.4 | * | * | * | * | * | 28.3 | * | * | * |
| **E365/11_02** | Negative | * | 29.4 | * | * | * | * | * | 32.0 | * | * | * |
| **E498/11** | Negative | * | 28.3 | * | * | * | * | * | 29.3 | * | * | * |
| **E565/11** | Negative | * | 28.4 | * | * | * | * | * | 27.9 | * | * | * |
| **E627/11** | Negative | * | 26.1 | * | * | * | * | * | 27.2 | * | * | * |
| **E004/12_03** | Negative | * | 23.5 | * | * | * | * | * | * | 28.4 | * | * |
| **E126/11** | Negative | * | 24.5 | * | * | * | * | * | * | 29.2 | * | * |
| **E134/11** | Negative | * | 25.4 | * | * | * | * | * | * | 30.5 | * | * |
| **E155/11** | Negative | * | 28.3 | * | * | * | * | * | * | 30.3 | * | * |
| **E179/11** | Negative | * | 25.2 | * | * | * | * | * | * | 28.3 | * | * |
| **E190/11** | Negative | * | 26.0 | * | * | * | * | * | * | 29.4 | * | * |
| **E225/11** | Negative | * | 27.5 | * | * | * | * | * | * | 30.1 | * | * |
| **E261/11_02** | Negative | * | 26.1 | * | * | * | * | * | * | 29.8 | * | * |
| **E265/11** | Negative | * | 26.2 | * | * | * | * | * | * | 29.2 | * | * |
| **E276/11** | Negative | * | 23.5 | * | * | * | * | * | * | 27.1 | * | * |
| **E280/11** | Negative | * | 24.6 | * | * | * | * | * | * | 27.8 | * | * |
| **E288/11_01** | Negative | * | 26.5 | * | * | * | * | * | * | 29.9 | * | * |
| **E345/11** | Negative | * | 27.2 | * | * | * | * | * | * | 27.9 | * | * |
| **E348/11** | Negative | * | 23.4 | * | * | * | * | * | * | 26.7 | * | * |
| **E352/11_01** | Negative | * | 28.0 | * | * | * | * | * | * | 28.9 | * | * |
| **E430/11** | Negative | * | 22.5 | * | * | * | * | * | * | 26.7 | * | * |
| **E438/11** | Negative | * | 26.2 | * | * | * | * | * | * | 27.5 | * | * |
| **E443/11_02** | Negative | * | 26.0 | * | * | * | * | * | * | 36.9 | * | * |
| **E525/11** | Negative | * | 28.8 | * | * | * | * | * | * | 31.8 | * | * |
| **E637/11** | Negative | * | 28.0 | * | * | * | * | * | * | 27.7 | * | * |
| **E029/11_01** | Negative | * | 28.9 | * | * | * | * | * | * | * | 24.1 | * |



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| Laboratory Reference | VI | PI | AHSV RT-qPCR Cq | AHSV TS RT-qPCR Cq | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| E030/11 | Negative | * | 27.2 | * | * | * | * | * | * | * | 30.2 | * |
| E042/11 | Negative | * | 29.7 | * | * | * | * | * | * | * | 27.8 | * |
| E106/12 | Negative | * | 26.7 | * | * | * | * | * | * | * | 28.8 | * |
| E108/11 | Negative | * | 26.5 | * | * | * | * | * | * | * | 29.1 | * |
| E121/11 | Negative | * | 25.1 | * | * | * | * | * | * | * | 27.4 | * |
| E161/11 | Negative | * | 27.6 | * | * | * | * | * | * | * | 30.0 | * |
| E229/11 | Negative | * | 25.8 | * | * | * | * | * | * | * | 26.6 | * |
| E232/11 | Negative | * | 26.8 | * | * | * | * | * | * | * | 27.1 | * |
| E263/11 | Negative | * | 28.0 | * | * | * | * | * | * | * | 30.0 | * |
| E275/11 | Negative | * | 28.3 | * | * | * | * | * | * | * | 29.2 | * |
| E325/11 | Negative | * | 29.7 | * | * | * | * | * | * | * | 29.4 | * |
| E330/11 | Negative | * | 22.2 | * | * | * | * | * | * | * | 22.3 | * |
| E368/11 | Negative | * | 24.9 | * | * | * | * | * | * | * | 26.7 | * |
| E369/11 | Negative | * | 23.6 | * | * | * | * | * | * | * | 25.2 | * |
| E384/11 | Negative | * | 28.8 | * | * | * | * | * | * | * | 25.7 | * |
| E389/11_04 | Negative | * | 25.3 | * | * | * | * | * | * | * | 27.3 | * |
| E433/11 | Negative | * | 24.3 | * | * | * | * | * | * | * | 25.9 | * |
| E434/11 | Negative | * | 26.5 | * | * | * | * | * | * | * | 28.1 | * |
| E446/11_24 | Negative | * | 24.6 | * | * | * | * | * | * | * | 26.6 | * |
| E474/11_02 | Negative | * | 27.4 | * | * | * | * | * | * | * | 28.7 | * |
| E499/11 | Negative | * | 25.0 | * | * | * | * | * | * | * | 27.4 | * |
| E502/11 | Negative | * | 27.8 | * | * | * | * | * | * | * | 29.4 | * |
| E544/11 | Negative | * | 29.9 | * | * | * | * | * | * | * | 31.8 | * |
| E550/11 | Negative | * | 25.5 | * | * | * | * | * | * | * | 23.9 | * |
| E595/11_06 | Negative | * | 26.2 | * | * | * | * | * | * | * | 27.0 | * |
| E651/11 | Negative | * | 26.0 | * | * | * | * | * | * | * | 27.4 | * |
| E688/11 | Negative | * | 28.5 | * | * | * | * | * | * | * | 31.2 | * |
| E731/11 | Negative | * | 27.1 | * | * | * | * | * | * | * | 27.9 | * |
| E765/11_02 | Negative | * | 24.9 | * | * | * | * | * | * | * | 34.7 | * |
| E905/11_02 | Negative | * | 27.0 | * | * | * | * | * | * | * | 30.4 | * |
| E515/11_02 | Negative | * | 29.6 | * | * | * | * | * | * | * | * | 33.6 |
| E617/11 | Negative | * | 28.1 | * | * | * | * | * | * | * | * | 32.0 |
| E143/11_01 | Positive | 1 | 20.4 | 21.3 | * | * | * | * | * | * | * | * |
| E170/11_01 | Positive | 1 | 26.5 | 27.0 | * | * | * | * | * | * | * | * |
| E410/11 | Positive | 1 | 23.6 | 21.9 | * | * | * | * | * | * | * | * |
| E411/11_01 | Positive | 1 | 20.8 | 20.2 | * | * | * | * | * | * | * | * |
| E411/11_02 | Positive | 1 | 22.4 | 22.9 | * | * | * | * | * | * | * | * |
| E418/11 | Positive | 1 | 26.8 | 25.2 | * | * | * | * | * | * | * | * |
| E420/11 | Positive | 1 | 23.0 | 23.9 | * | * | * | * | * | * | * | * |
| E421/11 | Positive | 1 | 26.3 | 27.0 | * | * | * | * | * | * | * | * |
| E428/11 | Positive | 1 | 21.8 | 21.3 | * | * | * | * | * | * | * | * |
| E473/11 | Positive | 1 | 24.4 | 24.8 | * | * | * | * | * | * | * | * |
| E509/11 | Positive | 1 | 27.0 | 26.0 | * | * | * | * | * | * | * | * |
| E511/11 | Positive | 1 | 24.1 | 22.2 | * | * | * | * | * | * | * | * |
| E514/11 | Positive | 1 | 25.0 | 23.5 | * | * | * | * | * | * | * | * |
| WC019 | Positive | 1 | 25.9 | 26.8 | * | * | * | * | * | * | * | * |
| WC026 | Positive | 1 | 20.8 | 22.5 | * | * | * | * | * | * | * | * |
| WC061 | Positive | 1 | 25.8 | 27.0 | * | * | * | * | * | * | * | * |
| WC075 | Positive | 1 | 25.7 | 26.3 | * | * | * | * | * | * | * | * |
| WC107 | Positive | 1 | 25.8 | 26.9 | * | * | * | * | * | * | * | * |
| WC128 | Positive | 1 | 25.5 | 27.2 | * | * | * | * | * | * | * | * |
| WC139 | Positive | 1 | 30.0 | 28.4 | * | * | * | * | * | * | * | * |
| WC147 | Positive | 1 | 32.3 | 33.0 | * | * | * | * | * | * | * | * |
| WC160 | Positive | 1 | 24.6 | 25.4 | * | * | * | * | * | * | * | * |
| E059/12 | Positive | 2 | 26.0 | * | 30.7 | * | * | * | * | * | * | * |
| E079/11 | Positive | 2 | 28.0 | * | 26.9 | * | * | * | * | * | * | * |
| E097/12 | Positive | 2 | 26.3 | * | 30.8 | * | * | * | * | * | * | * |



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| Laboratory Reference | VI | PI | AHSV RT-qPCR Cq | AHSV TS RT-qPCR Cq | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| **E130/11** | Positive | 2 | 24.6 | * | 28.0 | * | * | * | * | * | * | * |
| **E153/11** | Positive | 2 | 24.5 | * | 28.5 | * | * | * | * | * | * | * |
| **E203/12** | Positive | 2 | 23.3 | * | 27.6 | * | * | * | * | * | * | * |
| **E204/12** | Positive | 2 | 25.4 | * | 28.8 | * | * | * | * | * | * | * |
| **E329/11** | Positive | 2 | 26.5 | * | 30.1 | * | * | * | * | * | * | * |
| **E505/11** | Positive | 2 | 22.3 | * | 25.1 | * | * | * | * | * | * | * |
| **E025/12** | Positive | 3 | 23.7 | * | * | 25.2 | * | * | * | * | * | * |
| **E063/12** | Positive | 3 | 24.8 | * | * | 27.2 | * | * | * | * | * | * |
| **E084/11** | Positive | 3 | 27.9 | * | * | 28.2 | * | * | * | * | * | * |
| **E092/12** | Positive | 3 | 25.3 | * | * | 35.6 | * | * | * | * | * | * |
| **E191/11** | Positive | 3 | 28.3 | * | * | 26.7 | * | * | * | * | * | * |
| **E163/11** | Positive | 4 | 21.4 | * | * | * | 28.2 | * | * | * | * | * |
| **E069/12_03** | Positive | 4 | 24.7 | * | * | * | 30.4 | * | * | * | * | * |
| **E073/12** | Positive | 4 | 27.3 | * | * | * | 33.8 | * | * | * | * | * |
| **E088/12** | Positive | 4 | 25.3 | * | * | * | 29.6 | * | * | * | * | * |
| **E089/12** | Positive | 4 | 22.3 | * | * | * | 27.8 | * | * | * | * | * |
| **E094/11** | Positive | 4 | 27.0 | * | * | * | 37.4 | * | * | * | * | * |
| **E098/12** | Positive | 4 | 25.9 | * | * | * | 32.6 | * | * | * | * | * |
| **E116/12** | Positive | 4 | 19.0 | * | * | * | 24.1 | * | * | * | * | * |
| **E290/11** | Positive | 4 | 24.3 | * | * | * | 28.1 | * | * | * | * | * |
| **E456/11** | Positive | 4 | 19.2 | * | * | * | 25.5 | * | * | * | * | * |
| **E466/11** | Positive | 4 | 26.9 | * | * | * | 32.1 | * | * | * | * | * |
| **E006/12** | Positive | 5 | 20.1 | * | * | * | * | 25.3 | * | * | * | * |
| **E107/11** | Positive | 5 | 23.7 | * | * | * | * | 28.2 | * | * | * | * |
| **E125/11** | Positive | 5 | 24.9 | * | * | * | * | 28.7 | * | * | * | * |
| **E184/11** | Positive | 5 | 26.0 | * | * | * | * | 28.9 | * | * | * | * |
| **E196/11** | Positive | 5 | 17.9 | * | * | * | * | 20.9 | * | * | * | * |
| **E224/11** | Positive | 5 | 25.1 | * | * | * | * | 25.1 | * | * | * | * |
| **E234/11** | Positive | 5 | 26.4 | * | * | * | * | 27.9 | * | * | * | * |
| **E247/11** | Positive | 5 | 20.6 | * | * | * | * | 23.7 | * | * | * | * |
| **E289/11** | Positive | 5 | 23.2 | * | * | * | * | 30.0 | * | * | * | * |
| **E299/11** | Positive | 5 | 20.6 | * | * | * | * | 22.1 | * | * | * | * |
| **E300/11** | Positive | 5 | 21.5 | * | * | * | * | 25.1 | * | * | * | * |
| **E035/11** | Positive | 6 | 32.9 | * | * | * | * | * | 31.3 | * | * | * |
| **E040/12** | Positive | 6 | 25.5 | * | * | * | * | * | 27.8 | * | * | * |
| **E063/11** | Positive | 6 | 29.9 | * | * | * | * | * | 24.2 | * | * | * |
| **E069/12_01** | Positive | 6 | 30.9 | * | * | * | * | * | 32.9 | * | * | * |
| **E141/11** | Positive | 6 | 26.8 | * | * | * | * | * | 29.6 | * | * | * |
| **E141/12_02** | Positive | 6 | 25.7 | * | * | * | * | * | 28.9 | * | * | * |
| **E146/11** | Positive | 6 | 25.0 | * | * | * | * | * | 28.6 | * | * | * |
| **E193/12** | Positive | 6 | 24.4 | * | * | * | * | * | 25.6 | * | * | * |
| **E195/11** | Positive | 6 | 27.2 | * | * | * | * | * | 28.6 | * | * | * |
| **E216/11_02** | Positive | 6 | 22.9 | * | * | * | * | * | 24.5 | * | * | * |
| **E252/11** | Positive | 6 | 21.3 | * | * | * | * | * | 21.4 | * | * | * |
| **E254/11** | Positive | 6 | 23.2 | * | * | * | * | * | 22.0 | * | * | * |
| **E283/11** | Positive | 6 | 24.2 | * | * | * | * | * | 28.6 | * | * | * |
| **E322/11** | Positive | 6 | 29.1 | * | * | * | * | * | 29.0 | * | * | * |
| **E323/11** | Positive | 6 | 25.9 | * | * | * | * | * | 25.0 | * | * | * |
| **E404/11** | Positive | 6 | 27.5 | * | * | * | * | * | 29.8 | * | * | * |
| **E414/11** | Positive | 6 | 22.5 | * | * | * | * | * | 22.3 | * | * | * |
| **E467/11** | Positive | 6 | 25.7 | * | * | * | * | * | 27.8 | * | * | * |
| **E471/11** | Positive | 6 | 23.6 | * | * | * | * | * | 22.9 | * | * | * |
| **E521/11_02** | Positive | 6 | 27.4 | * | * | * | * | * | 28.8 | * | * | * |
| **E085/11** | Positive | 7 | 25.6 | * | * | * | * | * | * | 26.2 | * | * |
| **E100/11** | Positive | 7 | 27.3 | * | * | * | * | * | * | 31.0 | * | * |
| **E106/11** | Positive | 7 | 21.5 | * | * | * | * | * | * | 26.7 | * | * |
| **E136/11** | Positive | 7 | 28.0 | * | * | * | * | * | * | 31.1 | * | * |



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| Laboratory Reference | VI | PI | AHSV RT-qPCR Cq | AHSV TS RT-qPCR Cq | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| **E139/11** | Positive | 7 | 24.3 | * | * | * | * | * | * | 29.1 | * | * |
| **E147/11** | Positive | 7 | 23.4 | * | * | * | * | * | * | 26.2 | * | * |
| **E172/11** | Positive | 7 | 24.4 | * | * | * | * | * | * | 27.8 | * | * |
| **E189/11** | Positive | 7 | 28.1 | * | * | * | * | * | * | 32.5 | * | * |
| **E256/11** | Positive | 7 | 20.1 | * | * | * | * | * | * | 24.7 | * | * |
| **E284/11** | Positive | 7 | 25.1 | * | * | * | * | * | * | 29.7 | * | * |
| **E302/11_01** | Positive | 7 | 25.3 | * | * | * | * | * | * | 29.8 | * | * |
| **E336/11** | Positive | 7 | 25.2 | * | * | * | * | * | * | 30.5 | * | * |
| **E373/11_05** | Positive | 7 | 19.4 | * | * | * | * | * | * | 23.1 | * | * |
| **E443/11_01** | Positive | 7 | 28.1 | * | * | * | * | * | * | 33.1 | * | * |
| **E447/11_01** | Positive | 7 | 21.3 | * | * | * | * | * | * | 25.4 | * | * |
| **E484/11** | Positive | 7 | 19.1 | * | * | * | * | * | * | 23.0 | * | * |
| **E009/12** | Positive | 8 | 28.0 | * | * | * | * | * | * | * | 31.4 | * |
| **E013/12_04** | Positive | 8 | 20.5 | * | * | * | * | * | * | * | 22.7 | * |
| **E043/11** | Positive | 8 | 28.1 | * | * | * | * | * | * | * | 27.6 | * |
| **E050/11** | Positive | 8 | 30.7 | * | * | * | * | * | * | * | 27.1 | * |
| **E064/12_01** | Positive | 8 | 23.6 | * | * | * | * | * | * | * | 25.3 | * |
| **E068/12** | Positive | 8 | 25.1 | * | * | * | * | * | * | * | 28.8 | * |
| **E070/12_02** | Positive | 8 | 25.0 | * | * | * | * | * | * | * | 28.1 | * |
| **E077/11** | Positive | 8 | 32.3 | * | * | * | * | * | * | * | 33.9 | * |
| **E078/11** | Positive | 8 | 25.8 | * | * | * | * | * | * | * | 25.0 | * |
| **E081/11_01** | Positive | 8 | 29.8 | * | * | * | * | * | * | * | 27.2 | * |
| **E088/11** | Positive | 8 | 24.2 | * | * | * | * | * | * | * | 26.2 | * |
| **E099/11** | Positive | 8 | 25.4 | * | * | * | * | * | * | * | 28.8 | * |
| **E111/11** | Positive | 8 | 28.6 | * | * | * | * | * | * | * | 32.6 | * |
| **E112/11** | Positive | 8 | 25.9 | * | * | * | * | * | * | * | 29.4 | * |
| **E125/12** | Positive | 8 | 25.9 | * | * | * | * | * | * | * | 28.2 | * |
| **E137/11** | Positive | 8 | 23.6 | * | * | * | * | * | * | * | 26.7 | * |
| **E162/11** | Positive | 8 | 20.3 | * | * | * | * | * | * | * | 24.4 | * |
| **E165/11** | Positive | 8 | 27.9 | * | * | * | * | * | * | * | 29.1 | * |
| **E167/11** | Positive | 8 | 25.5 | * | * | * | * | * | * | * | 28.3 | * |
| **E185/11** | Positive | 8 | 18.9 | * | * | * | * | * | * | * | 21.0 | * |
| **E186/11** | Positive | 8 | 25.1 | * | * | * | * | * | * | * | 26.4 | * |
| **E197/11** | Positive | 8 | 25.5 | * | * | * | * | * | * | * | 27.5 | * |
| **E205/11** | Positive | 8 | 19.6 | * | * | * | * | * | * | * | 22.3 | * |
| **E207/11** | Positive | 8 | 20.7 | * | * | * | * | * | * | * | 22.6 | * |
| **E212/11** | Positive | 8 | 24.3 | * | * | * | * | * | * | * | 26.3 | * |
| **E221/11** | Positive | 8 | 21.5 | * | * | * | * | * | * | * | 23.5 | * |
| **E227/11_01** | Positive | 8 | 25.7 | * | * | * | * | * | * | * | 28.0 | * |
| **E239/11** | Positive | 8 | 21.8 | * | * | * | * | * | * | * | 23.8 | * |
| **E245/11** | Positive | 8 | 24.5 | * | * | * | * | * | * | * | 25.6 | * |
| **E258/11** | Positive | 8 | 24.6 | * | * | * | * | * | * | * | 26.5 | * |
| **E264/11** | Positive | 8 | 29.4 | * | * | * | * | * | * | * | 32.1 | * |
| **E268/11** | Positive | 8 | 22.4 | * | * | * | * | * | * | * | 24.8 | * |
| **E285/11** | Positive | 8 | 24.9 | * | * | * | * | * | * | * | 26.8 | * |
| **E288/11_02** | Positive | 8 | 19.6 | * | * | * | * | * | * | * | 22.1 | * |
| **E319/11** | Positive | 8 | 26.2 | * | * | * | * | * | * | * | 28.3 | * |
| **E321/11** | Positive | 8 | 22.4 | * | * | * | * | * | * | * | 24.7 | * |
| **E328/11** | Positive | 8 | 27.7 | * | * | * | * | * | * | * | 28.6 | * |
| **E335/11** | Positive | 8 | 21.0 | * | * | * | * | * | * | * | 24.1 | * |
| **E363/11** | Positive | 8 | 25.0 | * | * | * | * | * | * | * | 29.3 | * |
| **E367/11** | Positive | 8 | 23.3 | * | * | * | * | * | * | * | 26.3 | * |
| **E371/11** | Positive | 8 | 27.6 | * | * | * | * | * | * | * | 29.0 | * |
| **E373/11_03** | Positive | 8 | 20.6 | * | * | * | * | * | * | * | 23.9 | * |
| **E375/11** | Positive | 8 | 24.4 | * | * | * | * | * | * | * | 27.8 | * |
| **E378/11** | Positive | 8 | 27.8 | * | * | * | * | * | * | * | 31.6 | * |
| **E385/11** | Positive | 8 | 20.9 | * | * | * | * | * | * | * | 24.1 | * |



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| Laboratory Reference | VI | PI | AHSV RT-qPCR Cq | AHSV TS RT-qPCR Cq | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| **E422/11_02** | Positive | 8 | 26.5 | * | * | * | * | * | * | * | 28.7 | * |
| **E423/11** | Positive | 8 | 21.4 | * | * | * | * | * | * | * | 23.5 | * |
| **E424/11** | Positive | 8 | 23.9 | * | * | * | * | * | * | * | 25.7 | * |
| **E435/11** | Positive | 8 | 24.5 | * | * | * | * | * | * | * | 30.2 | * |
| **E451/11** | Positive | 8 | 24.9 | * | * | * | * | * | * | * | 27.5 | * |
| **E472/11** | Positive | 8 | 24.5 | * | * | * | * | * | * | * | 24.6 | * |
| **E474/11_01** | Positive | 8 | 21.6 | * | * | * | * | * | * | * | 23.6 | * |
| **E486/11** | Positive | 8 | 27.7 | * | * | * | * | * | * | * | 29.2 | * |
| **E491/11** | Positive | 8 | 20.9 | * | * | * | * | * | * | * | 22.0 | * |
| **E515/11_01** | Positive | 8 | 25.5 | * | * | * | * | * | * | * | 26.0 | * |
| **E524/11** | Positive | 8 | 24.7 | * | * | * | * | * | * | * | 26.0 | * |
| **E526/11** | Positive | 8 | 25.0 | * | * | * | * | * | * | * | 31.6 | * |
| **E905/11_01** | Positive | 8 | 23.7 | * | * | * | * | * | * | * | 26.3 | * |
| **E209/11** | Positive | 9 | 25.2 | * | * | * | * | * | * | * | * | 32.4 |
| **E210/11_01** | Positive | 9 | 24.1 | * | * | * | * | * | * | * | * | 32.4 |
| **E210/11_02** | Positive | 9 | 22.8 | * | * | * | * | * | * | * | * | 30.8 |
| **E446/11_25** | Positive | 9 | 28.1 | * | * | * | * | * | * | * | * | 36.6 |



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# APPENDIX B

**B1: A summary of the Group Specific RT-qPCR (GS) and Type Specific RT-qPCR (TS) results over time for each of the weanlings in Treatment group I. The Cq value for each of the GS results is given, and the TS result indicating which AHS type was obtained is given below each corresponding positive GS result. N indicates that the Cq value for the GS PCR was > 37. The Minimum Cq per week and Median Cq value per week is given as well as the total number of positive weanlings on GS PCR per week. ND means no AHSV type could be detected. * indicates that a samples was not tested. The column highlighted in red indicates the week when OBP comb1 was given (types 1, 3 and 4) and the column highlighted in green indicates the week OBP comb2 (types 2, 6, 7 and 8) was given. The age of each of the weanlings is given in days (d), and is the age at the start of the study at the time of initial vaccination at week 0.**

| Horse | Age (d) | Test | Weeks post initial vaccination | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| 3 | 254 | GS | N | 35.0 | 31.7 | 28.2 | 24.3 | 26.6 | 28.5 | 31.6 | 29.9 | 32.7 | 34.5 | 33.7 | 33.1 | N | 33.3 | N | 33.8 |
| | | TS | | | 1,4 | 1 | 1,3 | 1,3 | 1,3 | 3 | 1,3 | 3 | | | | | | | |
| 7 | 202 | GS | N | 34.5 | 27.8 | 28.8 | 28.3 | 25.3 | 28.3 | 28.9 | 28.3 | 34.4 | 35.6 | N | 34.4 | 33.9 | * | N | 34.9 |
| | | TS | | | 1 | 1 | 1 | 1 | 1 | 1,4 | 1,4 | | | | | | | | |
| 8 | 200 | GS | N | N | N | 27.5 | 27.4 | 31.6 | 33.0 | N | 28.7 | 31.0 | 36.9 | N | N | N | * | N | N |
| | | TS | | | | 1 | 1 | 1 | 1 | | 1 | 1 | | | | | | | |
| 14 | 239 | GS | N | 33.2 | 30.5 | 27.5 | 22.3 | 25.2 | 27.4 | 30.6 | 28.4 | 30.7 | 32.0 | 33.0 | 31.9 | 34.2 | * | N | 32.4 |
| | | TS | | | 1 | 1 | 1,3 | 1,3 | 1,3 | 1,3 | 1,3 | 1,3 | ND | | 1,3 | | | | ND |
| 27 | 242 | GS | N | 31.1 | 32.4 | 29.3 | 29.9 | 33.3 | 33.2 | 36.9 | 35.6 | N | 36.6 | 36.2 | N | N | * | N | N |
| | | TS | | 1 | 1 | 1 | 1 | | | | | | | | | | | | |
| 30 | 212 | GS | N | N | N | N | N | N | N | N | N | N | N | N | N | N | * | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 38 | 217 | GS | N | N | N | N | N | N | N | N | N | N | N | N | N | N | * | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 39 | 218 | GS | N | N | N | 27.9 | 29.6 | 32.2 | 32.7 | 36.9 | 33.4 | 36.3 | 36.1 | N | N | N | * | N | 36.7 |
| | | TS | | | | 1 | 1 | 1 | 1 | | | | | | | | | | |
| 45 | 239 | GS | N | N | 33.8 | 33.7 | 34.4 | N | N | N | 31.9 | 34.6 | N | N | 36.6 | N | N | N | N |
| | | TS | | | | | | | | | 3 | | | | | | | | |
| 51 | 250 | GS | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 53 | 231 | GS | N | N | N | 35.5 | 32.6 | 35.6 | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | ND | | | | | | | | | | | | |
| **Min. Age** | **200** | **Min. Cq** | **N** | **31.1** | **27.8** | **27.5** | **22.3** | **25.2** | **27.4** | **28.9** | **28.3** | **30.7** | **32.0** | **33.0** | **31.9** | **33.9** | **33.3** | **N** | **32.4** |
| **Max. Age** | **254** | **Median Cq** | **N** | **N** | **N** | **29.3** | **29.9** | **33.3** | **33.2** | **N** | **33.4** | **36.3** | **36.9** | **N** | **N** | **N** | **N** | **N** | **N** |
| **Ave. Age** | **228** | **Total Pos** | **0** | **4** | **5** | **8** | **8** | **7** | **6** | **5** | **7** | **6** | **6** | **3** | **4** | **2** | **1** | **0** | **4** |



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**Table B2: A summary of the Group Specific RT-qPCR (GS) and Type Specific RT-qPCR (TS) results over time for each of the weanlings in Treatment group II. The Cq value for each of the GS results is given, and the TS result indicating which AHS type was obtained is given below each corresponding positive GS result. N indicates that the Cq value for the GS PCR was > 37. The Minimum Cq per week and Median Cq value per week is given as well as the total number of positive weanlings on GS PCR per week. ND means no AHSV type could be detected. * indicates that a samples was not tested. The column highlighted in red indicates the week when OBP comb1 was given (types 1, 3 and 4) and the column highlighted in green indicates the week OBP comb2 (types 2, 6, 7 and 8) was given. The age of each of the weanlings is given in days (d), and is the age at the start of the study at the time of initial vaccination at week 0.**

| Horse | Age (d) | Test | Weeks post initial vaccination | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| 1 | 284 | GS | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 9 | 269 | GS | N | 35.8 | 28.4 | 30.2 | 33.8 | 33.9 | 35.0 | 37.0 | 36.0 | 36.3 | N | N | N | N | 36.0 | N | N |
| | | TS | | | 8 | 8 | | | | | | | | | | | | | |
| 16 | 212 | GS | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 25 | 259 | GS | N | 35.5 | 27.8 | 30.7 | 33.4 | 32.5 | 33.0 | 34.8 | 35.2 | 36.3 | 32.2 | 33.9 | 36.2 | N | 35.0 | N | 37.0 |
| | | TS | | | 8 | 8 | | 8 | ND | | | | | | | | | | |
| 28 | 205 | GS | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 29 | 291 | GS | N | N | N | N | N | 33.5 | 28.7 | 30.7 | 31.6 | 33.9 | 35.0 | 34.5 | 34.7 | 36.9 | 34.5 | N | 35.3 |
| | | TS | | | | | | | ND | ND | 2 | | | | | | | | |
| 37 | 262 | GS | N | N | 28.5 | 30.3 | 33.3 | 32.8 | 29.9 | 31.8 | 35.1 | 35.9 | N | 35.1 | 35.9 | N | 36.9 | N | 35.0 |
| | | TS | | | 8 | 8 | | 8 | ND | 8 | | | | | | | | | |
| 41 | 263 | GS | N | N | N | 35.0 | 36.7 | 33.2 | 31.4 | 34.7 | N | N | 36.7 | 36.7 | N | N | N | N | N |
| | | TS | | | | | | | ND | | | | | | | | | | |
| 42 | 290 | GS | N | N | N | N | N | N | 36.2 | N | N | 33.9 | 34.5 | 36.6 | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 44 | 236 | GS | N | N | N | N | N | N | 36.1 | N | 33.4 | 36.7 | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 48 | 280 | GS | N | N | 36.4 | 35.2 | N | N | N | 34.5 | N | N | N | 35.7 | 33.7 | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| **Min. Age** | **205** | **Min. Cq** | **N** | **35.5** | **27.8** | **30.2** | **33.3** | **32.5** | **28.7** | **30.7** | **31.6** | **33.9** | **32.2** | **33.9** | **33.7** | **36.9** | **34.5** | **N** | **35.0** |
| **Max. Age** | **291** | **Median Cq** | **N** | **N** | **N** | **N** | **N** | **N** | **36.1** | **37.0** | **N** | **36.7** | **N** | **36.7** | **N** | **N** | **N** | **N** | **N** |
| **Ave. Age** | **259** | **Total Pos** | **0** | **2** | **4** | **5** | **4** | **4** | **5** | **6** | **5** | **6** | **4** | **6** | **4** | **1** | **4** | **0** | **3** |



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**Table B3: A summary of the Group Specific RT-qPCR (GS) and Type Specific RT-qPCR (TS) results over time for each of the weanlings in Treatment group III. The Cq value for each of the GS results is given, and the TS result indicating which AHS type was obtained is given below each corresponding positive GS result. N indicates that the Cq value for the GS PCR was > 37. The Minimum Cq per week and Median Cq value per week is given as well as the total number of positive weanlings on GS PCR per week. ND means no AHSV type could be detected. * indicates that a samples was not tested. The column highlighted in red indicates the week when OBP comb1 was given (types 1, 3 and 4) and the column highlighted in green indicates the week OBP comb2 (types 2, 6, 7 and 8) was given. The age of each of the weanlings is given in days (d), and is the age at the start of the study at the time of initial vaccination at week 0.**

| Horse | Age (d) | Test | Weeks post initial vaccination | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| 5 | 231 | GS | N | 36.7 | 35.2 | 36.9 | 36.0 | 30.0 | 35.4 | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | 1 | | | | | | | | | | | |
| 11 | 227 | GS | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 20 | 250 | GS | N | N | N | 36.4 | 35.0 | 36.4 | 36.0 | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 26 | 202 | GS | N | N | N | N | N | N | 36.7 | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 34 | 257 | GS | N | 32.9 | 28.8 | 28.6 | 29.2 | 28.5 | 34.5 | 36.1 | 33.7 | 34.8 | N | N | N | N | N | N | N |
| | | TS | | 1 | 1 | 1 | 1,3 | 1,3 | | | | | | | | | | | |
| 40 | 249 | GS | N | N | N | N | N | 31.0 | 30.3 | 32.4 | 35.5 | N | N | N | N | N | N | N | N |
| | | TS | | | | | | 3 | 3 | 3 | | | | | | | | | |
| 43 | 243 | GS | N | N | N | 31.8 | 30.9 | 31.5 | 35.4 | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | 3 | 3 | 3 | | | | | | | | | | | |
| 55 | 250 | GS | N | N | 35.3 | N | N | 33.6 | 33.0 | 34.3 | N | N | N | 35.0 | N | N | N | N | N |
| | | TS | | | | | | | 1 | | | | | | | | | | |
| 57 | 237 | GS | N | N | 32.8 | 30.5 | 33.7 | 28.7 | 33.3 | N | N | N | N | N | N | N | N | N | 32.8 |
| | | TS | | | 1 | 1 | | 1 | | | | | | | | | | | ND |
| 59 | 232 | GS | N | N | 33.8 | 37.0 | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 62 | 208 | GS | N | N | N | N | 36.7 | 31.8 | 36.8 | N | N | 34.3 | 35.2 | N | N | N | N | N | N |
| | | TS | | | | | | 1 | | | | | | | | | | | |
| **Min. Age** | **202** | **Min. Cq** | **N** | **32.9** | **28.8** | **28.6** | **29.2** | **28.5** | **30.3** | **32.4** | **33.7** | **34.3** | **35.2** | **35.0** | **N** | **N** | **N** | **N** | **32.8** |
| **Max. Age** | **257** | **MedianCq** | **N** | **N** | **N** | **N** | **36.7** | **31.8** | **35.4** | **N** | **N** | **N** | **N** | **N** | **N** | **N** | **N** | **N** | **N** |
| **Ave. Age** | **235** | **Total Pos** | **0** | **2** | **5** | **6** | **6** | **8** | **9** | **3** | **2** | **2** | **1** | **1** | **0** | **0** | **0** | **0** | **1** |



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**Table B4: A summary of the Group Specific RT-qPCR (GS) and Type Specific RT-qPCR (TS) results over time for each of the weanlings in Treatment group IV. The Cq value for each of the GS results is given, and the TS result indicating which AHS type was obtained is given below each corresponding positive GS result. N indicates that the Cq value for the GS PCR was > 37. The Minimum Cq per week and Median Cq value per week is given as well as the total number of positive weanlings on GS PCR per week. ND means no AHSV type could be detected. * indicates that a samples was not tested. The column highlighted in red indicates the week when OBP comb1 LAV vaccine was given (types 1, 3 and 4) and the column highlighted in green indicates the week OBP comb2 (types 2, 6, 7 and 8) was given. The age of each of the weanlings is given in days (d), and is the age at the start of the study at the time of initial vaccination at week 0.**

| Horse | Age (d) | Test | Weeks post initial vaccination | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| 4 | 279 | GS | N | N | 28.9 | 28.8 | 33.6 | 33.7 | 35.5 | 36.7 | N | N | N | N | N | 35.6 | 31.1 | 34.6 | 32.9 |
| | | TS | | | 8 | 2,8 | | | | | | | | | | | ND | | 1,3 |
| 13 | 225 | GS | N | N | 27.9 | 28.5 | 31.7 | 31.8 | 33.9 | 35.6 | 36.1 | N | 35.1 | 30.5 | N | 35.9 | N | 34.5 | 32.2 |
| | | TS | | | 8 | 8 | 8 | 8 | | | | | | ND | | | | | ND |
| 15 | 240 | GS | N | N | 30.1 | 29.8 | 33.1 | 26.8 | 28.4 | 33.1 | 36.5 | N | 33.1 | 29.0 | 37.0 | 35.5 | N | N | 36.4 |
| | | TS | | | 6,8 | 6,8 | | 2,8 | 2 | | | | | 8 | | | | | |
| 22 | 273 | GS | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 23 | 273 | GS | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 32 | 288 | GS | N | N | N | N | N | 34.9 | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 33 | 234 | GS | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 35 | 211 | GS | N | N | N | N | N | N | N | N | N | N | N | N | 36.1 | 35.7 | N | N | N |
| | | TS | | | | | | | | | | | | | | | | | |
| 46 | 295 | GS | N | N | N | N | N | 35.2 | 32.7 | N | N | N | N | N | N | N | N | N | N |
| | | TS | | | | | | | ND | | | | | | | | | | |
| 50 | 272 | GS | N | N | 36.9 | 30.6 | 30.8 | 32.6 | N | N | N | 36.3 | N | N | N | N | N | N | 35.9 |
| | | TS | | | | ND | ND | ND | | | | | | | | | | | |
| 52 | 282 | GS | N | 35.2 | 27.1 | 28.2 | 31.8 | 31.7 | 30.2 | 31.9 | 34.6 | 35.7 | N | 28.5 | 32.5 | 36.9 | N | N | 36.7 |
| | | TS | | | 8 | 8 | 8 | 8 | 2,8 | 8 | | | | 2 | ND | | | | |
| **Min. Age** | **211** | **Min. Cq** | **N** | **35.2** | **27.1** | **28.2** | **30.8** | **26.8** | **28.4** | **31.9** | **34.6** | **35.7** | **33.1** | **28.5** | **32.5** | **35.5** | **31.1** | **34.5** | **32.2** |
| **Max. Age** | **295** | **MedianCq** | **N** | **N** | **N** | **N** | **N** | **34.9** | **N** | **N** | **N** | **N** | **N** | **N** | **N** | **N** | **N** | **N** | **N** |
| **Ave. Age** | **236** | **Total Pos** | **0** | **1** | **5** | **5** | **5** | **7** | **5** | **4** | **3** | **2** | **2** | **3** | **3** | **5** | **1** | **2** | **5** |



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# APPENDIX C

**Table: SNT results for each of the weanlings in each of the 4 groups. For each weanling (HorseNo) the group number is given (Group I to IV), the Week number after the start of the study (Week 0 being the first week of sample collection and vaccination), and the SNT titre result given for each of the 9 AHSV types.**

| HorseNo | Group | Week | AHSV 1 | AHSV 2 | AHSV 3 | AHSV 4 | AHSV 5 | AHSV 6 | AHSV 7 | AHSV 8 | AHSV 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | Group I | 4 | 160 | 0 | 0 | 40 | 0 | 0 | 0 | 10 | 10 |
| 3 | Group I | 8 | 224 | 10 | >320 | 0 | 20 | 56 | 40 | 10 | 40 |
| 3 | Group I | 12 | 160 | 0 | >320 | 20 | 10 | 20 | 20 | 0 | 20 |
| 3 | Group I | 16 | 56 | 0 | >320 | 14 | 20 | 40 | 28 | 14 | 80 |
| 7 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | Group I | 4 | 160 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | Group I | 8 | >320 | 28 | 0 | >320 | 20 | 20 | 10 | 10 | 28 |
| 7 | Group I | 12 | 160 | 0 | 0 | 112 | 0 | 10 | 0 | 0 | 10 |
| 7 | Group I | 16 | >320 | 112 | 14 | 80 | 10 | 28 | 14 | 40 | 80 |
| 8 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | Group I | 4 | 320 | 0 | 0 | 0 | 0 | 14 | 0 | 0 | 0 |
| 8 | Group I | 8 | 160 | 14 | 0 | 10 | 10 | 14 | 0 | 0 | 14 |
| 8 | Group I | 12 | >320 | 56 | 0 | 0 | 0 | 14 | 10 | 0 | 40 |
| 8 | Group I | 16 | >320 | 20 | 0 | 10 | 0 | 20 | 10 | 0 | 28 |
| 14 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | Group I | 4 | 80 | 0 | 0 | 80 | 0 | 20 | 0 | 0 | 20 |
| 14 | Group I | 8 | 320 | 10 | >320 | 80 | 40 | 28 | 80 | 14 | 40 |
| 14 | Group I | 12 | 320 | 0 | 224 | 20 | 20 | 40 | 40 | 10 | 40 |
| 14 | Group I | 16 | 112 | 0 | 224 | 14 | 20 | 20 | 20 | 10 | 28 |
| 27 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27 | Group I | 4 | 160 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27 | Group I | 8 | 80 | 10 | 0 | 0 | 0 | 14 | 0 | 0 | 10 |
| 27 | Group I | 12 | >320 | 40 | 0 | 0 | 0 | 20 | 0 | 0 | 80 |
| 27 | Group I | 16 | >320 | 10 | 0 | 0 | 0 | 20 | 0 | 0 | 40 |
| 30 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | Group I | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | Group I | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | Group I | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | Group I | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38 | Group I | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 38 | Group I | 8 | 10 | 0 | 0 | 160 | 0 | 0 | 0 | 0 | 0 |
| 38 | Group I | 12 | 0 | 0 | 0 | 28 | 0 | 0 | 0 | 0 | 0 |
| 38 | Group I | 16 | 0 | 0 | 0 | 14 | 0 | 0 | 0 | 0 | 0 |
| 39 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | Group I | 4 | 112 | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 10 |
| 39 | Group I | 8 | 320 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| 39 | Group I | 12 | 320 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 39 | Group I | 16 | 160 | 0 | 0 | 0 | 0 | 14 | 0 | 0 | 0 |
| 45 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | Group I | 4 | 0 | 0 | 0 | 14 | 0 | 0 | 0 | 0 | 0 |
| 45 | Group I | 8 | 0 | 0 | 0 | 80 | 0 | 0 | 0 | 0 | 0 |
| 45 | Group I | 12 | 0 | 0 | 56 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45 | Group I | 16 | 0 | 0 | 28 | 160 | 0 | 10 | 0 | 0 | 0 |
| 51 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 51 | Group I | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 51 | Group I | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 51 | Group I | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 51 | Group I | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 53 | Group I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 53 | Group I | 4 | 112 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 53 | Group I | 8 | 320 | 0 | 0 | 14 | 10 | 14 | 0 | 0 | 0 |
| 53 | Group I | 12 | 80 | 0 | 0 | 20 | 0 | 0 | 0 | 0 | 0 |
| 53 | Group I | 16 | 80 | 0 | 0 | 14 | 0 | 10 | 0 | 0 | 0 |



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| HorseNo | Group | Week | AHSV 1 | AHSV 2 | AHSV 3 | AHSV 4 | AHSV 5 | AHSV 6 | AHSV 7 | AHSV 8 | AHSV 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | Group II | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | Group II | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | Group II | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 | Group II | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | Group II | 4 | 20 | 0 | 0 | 0 | >320 | 20 | 0 | >320 | 20 |
| 9 | Group II | 8 | 20 | 0 | 0 | 0 | 28 | 14 | 0 | 320 | 10 |
| 9 | Group II | 12 | 20 | 0 | 0 | 0 | 20 | 10 | 0 | 160 | 14 |
| 9 | Group II | 16 | 20 | 0 | 0 | 0 | 28 | 10 | 0 | 80 | 0 |
| 16 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | Group II | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | Group II | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | Group II | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | Group II | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25 | Group II | 4 | 20 | 0 | 0 | 0 | 56 | 20 | 0 | 320 | 20 |
| 25 | Group II | 8 | 56 | 28 | 0 | 0 | 112 | 60 | 0 | 160 | 20 |
| 25 | Group II | 12 | 160 | >320 | 0 | 10 | 56 | 160 | 0 | >320 | 40 |
| 25 | Group II | 16 | 80 | 160 | 0 | 0 | 28 | 20 | 0 | 160 | 28 |
| 28 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | Group II | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | Group II | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | Group II | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | Group II | 16 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 29 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 29 | Group II | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 29 | Group II | 8 | 56 | 112 | 0 | 0 | 0 | 20 | 0 | 0 | 0 |
| 29 | Group II | 12 | 80 | 56 | 0 | 0 | 0 | 14 | 0 | 0 | 0 |
| 29 | Group II | 16 | 80 | 28 | 0 | 0 | 0 | 14 | 0 | 0 | 0 |
| 37 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 37 | Group II | 4 | 10 | 0 | 0 | 0 | 40 | 20 | 0 | 320 | 10 |
| 37 | Group II | 8 | 20 | 0 | 0 | 0 | >320 | 112 | 0 | >320 | 40 |
| 37 | Group II | 12 | 10 | 0 | 0 | 0 | 80 | 80 | 0 | >320 | 28 |
| 37 | Group II | 16 | 20 | 0 | 0 | 0 | 40 | 40 | 0 | 320 | 20 |
| 41 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 41 | Group II | 4 | 0 | 0 | 0 | 0 | 0 | 14 | 0 | 28 | 0 |
| 41 | Group II | 8 | 10 | 14 | 0 | 0 | 14 | 14 | 0 | 56 | 0 |
| 41 | Group II | 12 | 40 | 40 | 0 | 0 | 10 | 14 | 0 | 112 | 0 |
| 41 | Group II | 16 | 28 | 40 | 0 | 0 | 10 | 20 | 0 | 80 | 0 |
| 42 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 42 | Group II | 4 | 0 | 0 | 320 | 0 | 0 | 0 | 0 | 0 | 0 |
| 42 | Group II | 8 | 20 | 0 | 0 | 0 | 56 | 28 | 0 | 40 | 10 |
| 42 | Group II | 12 | 10 | 0 | 10 | 0 | 80 | 40 | 0 | >320 | 40 |
| 42 | Group II | 16 | 10 | 0 | 0 | 0 | 28 | 28 | 0 | 320 | 20 |
| 44 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 44 | Group II | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 44 | Group II | 8 | 10 | 0 | 0 | 80 | 0 | 10 | 0 | 0 | 0 |
| 44 | Group II | 12 | 160 | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 |
| 44 | Group II | 16 | 160 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| 48 | Group II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 48 | Group II | 4 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 40 | 0 |
| 48 | Group II | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 |
| 48 | Group II | 12 | 20 | 0 | 14 | 0 | 80 | 20 | 0 | >320 | 10 |
| 48 | Group II | 16 | 0 | 0 | 0 | 0 | 10 | 14 | 0 | >320 | 10 |



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| HorseNo | Group | Week | AHSV 1 | AHSV 2 | AHSV 3 | AHSV 4 | AHSV 5 | AHSV 6 | AHSV 7 | AHSV 8 | AHSV 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Group III | 4 | 0 | 0 | 0 | 14 | 0 | 0 | 0 | 0 | 0 |
| 5 | Group III | 8 | 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Group III | 12 | 320 | 0 | 0 | >320 | 0 | 10 | 0 | 0 | 10 |
| 5 | Group III | 16 | 80 | 0 | 0 | 160 | 0 | 14 | 0 | 10 | 10 |
| 11 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | Group III | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | Group III | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | Group III | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | Group III | 16 | 20 | 0 | 0 | 0 | 0 | 56 | 0 | 0 | 0 |
| 20 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | Group III | 4 | NT | NT | NT | NT | NT | NT | NT | NT | NT |
| 20 | Group III | 8 | 160 | 0 | 0 | 20 | 0 | 10 | 0 | 0 | 0 |
| 20 | Group III | 12 | >320 | 28 | 0 | 56 | 0 | 20 | 0 | 10 | 10 |
| 20 | Group III | 16 | 320 | 20 | 0 | 40 | 10 | 28 | 0 | 20 | 28 |
| 26 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | Group III | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | Group III | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | Group III | 12 | 10 | 0 | 0 | 0 | 0 | 20 | 0 | 0 | 0 |
| 26 | Group III | 16 | 20 | 0 | 0 | 10 | 0 | 20 | 0 | 0 | 0 |
| 34 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 34 | Group III | 4 | 320 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 34 | Group III | 8 | 112 | 0 | 112 | 0 | 10 | 20 | 10 | 0 | 14 |
| 34 | Group III | 12 | 56 | 0 | 224 | >320 | 10 | 56 | 20 | 20 | 56 |
| 34 | Group III | 16 | 80 | 0 | 28 | 80 | 10 | 20 | 14 | 20 | 28 |
| 40 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 40 | Group III | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 40 | Group III | 8 | 10 | 0 | 224 | 0 | 0 | 14 | 10 | 0 | 10 |
| 40 | Group III | 12 | 0 | 0 | >320 | 0 | 0 | 20 | 0 | 0 | 10 |
| 40 | Group III | 16 | 0 | 0 | 112 | 0 | 0 | 28 | 0 | 0 | 14 |
| 43 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 43 | Group III | 4 | 10 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 10 |
| 43 | Group III | 8 | 10 | 0 | 56 | 0 | 0 | 0 | 0 | 0 | 0 |
| 43 | Group III | 12 | 0 | 0 | 224 | 0 | 0 | 0 | 0 | 0 | 0 |
| 43 | Group III | 16 | 0 | 0 | 28 | 0 | 0 | 0 | 0 | 0 | 0 |
| 55 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 55 | Group III | 4 | 0 | 0 | 0 | 14 | 0 | 0 | 0 | 0 | 0 |
| 55 | Group III | 8 | >320 | 0 | 0 | 40 | 10 | 20 | 0 | 0 | 0 |
| 55 | Group III | 12 | >320 | 10 | 0 | >320 | 10 | 20 | 0 | 20 | 20 |
| 55 | Group III | 16 | 112 | 0 | 0 | 160 | 10 | 20 | 0 | 10 | 20 |
| 57 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 57 | Group III | 4 | 112 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 57 | Group III | 8 | >320 | 10 | 0 | 0 | 0 | 10 | 0 | 0 | 10 |
| 57 | Group III | 12 | >320 | 14 | 14 | 0 | 0 | 10 | 0 | 0 | 10 |
| 57 | Group III | 16 | 160 | 10 | 0 | 0 | 0 | 20 | 0 | 10 | 20 |
| 59 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 59 | Group III | 4 | 0 | 0 | 0 | 10 | 0 | 0 | 0 | 0 | 0 |
| 59 | Group III | 8 | 10 | 0 | 0 | 40 | 0 | 0 | 0 | 0 | 0 |
| 59 | Group III | 12 | 0 | 0 | 0 | 20 | 0 | 10 | 0 | 0 | 0 |
| 59 | Group III | 16 | 20 | 0 | 0 | 20 | 0 | 20 | 0 | 10 | 0 |
| 62 | Group III | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 62 | Group III | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 62 | Group III | 8 | 160 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 62 | Group III | 12 | >320 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| 62 | Group III | 16 | 112 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |



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| HorseNo | Group | Week | AHSV 1 | AHSV 2 | AHSV 3 | AHSV 4 | AHSV 5 | AHSV 6 | AHSV 7 | AHSV 8 | AHSV 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | Group IV | 4 | 0 | 0 | 0 | 0 | 40 | 10 | 0 | >320 | 10 |
| 4 | Group IV | 8 | 20 | 0 | 0 | 0 | 56 | 20 | 0 | 320 | 10 |
| 4 | Group IV | 12 | 0 | 0 | 0 | 0 | 20 | 14 | 0 | 80 | 0 |
| 4 | Group IV | 16 | 320 | 20 | 0 | 56 | 320 | 28 | 0 | >320 | 160 |
| 13 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 13 | Group IV | 4 | 10 | 0 | 0 | 0 | 160 | 14 | 0 | >320 | 20 |
| 13 | Group IV | 8 | 20 | 0 | 0 | 0 | 56 | 14 | 0 | >320 | 10 |
| 13 | Group IV | 12 | 160 | 56 | 0 | 0 | 320 | 40 | 0 | >320 | 20 |
| 13 | Group IV | 16 | 160 | 320 | 0 | 0 | 80 | 20 | 20 | 320 | 56 |
| 15 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | Group IV | 4 | 0 | 0 | 0 | 0 | 20 | 160 | 0 | 80 | 20 |
| 15 | Group IV | 8 | 40 | 56 | 0 | 0 | 40 | 160 | 0 | 112 | 40 |
| 15 | Group IV | 12 | 90 | 80 | 112 | 0 | >320 | >320 | 10 | >320 | 160 |
| 15 | Group IV | 16 | 56 | 28 | 28 | 0 | 80 | 80 | 0 | >320 | 80 |
| 22 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | Group IV | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | Group IV | 8 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | Group IV | 12 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | Group IV | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | Group IV | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 23 | Group IV | 8 | 10 | 0 | 0 | 0 | 0 | 59 | 0 | 0 | 0 |
| 23 | Group IV | 12 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| 23 | Group IV | 16 | 10 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| 32 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | Group IV | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | Group IV | 8 | 10 | 0 | 0 | 0 | 40 | 0 | 0 | 112 | 0 |
| 32 | Group IV | 12 | 0 | 0 | 14 | 0 | 40 | 20 | 0 | 160 | 10 |
| 32 | Group IV | 16 | 0 | 0 | 0 | 0 | 40 | 10 | 0 | >320 | 0 |
| 33 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 33 | Group IV | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 33 | Group IV | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 33 | Group IV | 12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 33 | Group IV | 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 35 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 35 | Group IV | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 35 | Group IV | 8 | 10 | 0 | 0 | 0 | 0 | 28 | 0 | 20 | 0 |
| 35 | Group IV | 12 | 0 | 0 | 0 | 0 | 0 | 28 | 0 | 56 | 0 |
| 35 | Group IV | 16 | 0 | 0 | 0 | 0 | 20 | 20 | 0 | 80 | 10 |
| 46 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 46 | Group IV | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 46 | Group IV | 8 | 320 | 28 | 0 | 0 | 0 | 20 | 0 | 0 | 0 |
| 46 | Group IV | 12 | 80 | 20 | 0 | 0 | 0 | 20 | 0 | 0 | 10 |
| 46 | Group IV | 16 | 80 | 224 | 0 | 0 | 0 | 20 | 0 | 0 | 10 |
| 50 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 50 | Group IV | 4 | 10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 50 | Group IV | 8 | 40 | 28 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| 50 | Group IV | 12 | 40 | 40 | 0 | 0 | 20 | 20 | 0 | 80 | 0 |
| 50 | Group IV | 16 | 80 | 20 | 0 | 0 | 56 | 28 | 0 | 160 | 40 |
| 52 | Group IV | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 52 | Group IV | 4 | 10 | 0 | 0 | 0 | 160 | 10 | 0 | 320 | 0 |
| 52 | Group IV | 8 | 112 | 80 | 0 | 0 | 320 | 40 | 0 | 320 | 14 |
| 52 | Group IV | 12 | 160 | >320 | 0 | 10 | 80 | 80 | 20 | 320 | 56 |
| 52 | Group IV | 16 | 80 | 320 | 0 | 0 | 40 | 40 | 10 | 40 | 20 |



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**Technical Appendix 1. Metadata for each of the 39 AHSV type 1 field isolates from the 2004 Stellenbosch, 2011 Mamre, 2014 Porterville and 2014 Robertson outbreaks of AHS in the AHS Controlled Area of the Western Cape Province of South Africa. Each record includes the Strain Name of the isolate as submitted to GenBank, the Abbreviated Strain Name, a hyperlink to the Biosample details for the isolate, a hyperlink to the GenBank accession numbers for the isolate, the average coverage per nucleotide for the consensus sequences, the animal name or laboratory name from which the sample or material was sourced, reference numbers for publications that have previously described these cases or isolates, the genotype group of the field isolate, the outcome of the case, the type off samples submitted for examination, the date that the samples were collected and the vaccination status of the donor horse.**

| Strain Name[a] | Abbreviated Strain Name[b] | BioSample Number | Assession Numbers | Coverage | Animal Name/Source | Reference | Genotype Group | Outcome | Sample | Sampled | Vaccination[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Elsenburg-E04001 | 1/E.cab-tc/ZAF/04/Elb-E040019 | SAMN04038467 | KT715641 - KT715650 | 101369 | Laura | [23, 24, 25] | 1a | Died | Organs | 22/02/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Elsenburg-E04002 | 1/E.cab-tc/ZAF/04/Elb-E040020 | SAMN03774773 | KT186897 - KT186906 | 3823 | Michelle | [23, 24, 25] | 1a | Died | Organs | 22/02/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Elsenburg-E04002 | 1/E.cab-tc/ZAF/04/Elb-E040021 | SAMN03774774 | KT186907 - KT186916 | 11093 | Mara | [23, 24, 25] | 1a | Died | Blood | 24/02/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Daktari-E040029 | 1/E.cab-tc/ZAF/04/Dkt-E040029 | SAMN03774771 | KT186917 - KT186926 | 9984 | Murphy Brown | [23, 24, 25] | 1a | Died | Blood | 28/02/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Troughend-E040031 | 1/E.cab-tc/ZAF/04/Tgd-E040031 | SAMN03774781 | KT186927 - KT186936 | 5223 | SA Saddlehorse 1 | [23, 24, 25] | 1b | Died | Organs | 26/02/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Elsenburg-E040034 | 1/E.cab-tc/ZAF/04/Elb-E040034 | SAMN03774775 | KT186937 - KT186946 | 13476 | Mandy | [23, 25] | 1b | Recovered | Blood | 26/02/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Troughend-E040039 | 1/E.cab-tc/ZAF/04/Tgd-E040039 | SAMN03774782 | KT186947 - KT186956 | 9825 | Melody Fire | [23, 25] | 1b | Recovered | Blood | 03/03/2004 | Vaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Avontuur-E040043 | 1/E.cab-tc/ZAF/04/Avt-E040043 | SAMN03774767 | KT186957 - KT186966 | 4683 | Bow Street Bell | [23, 24, 25] | 1b | Euthanased | Blood | 04/03/2004 | Vaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Avontuur-E040048 | 1/E.cab-tc/ZAF/04/Avt-E040048 | SAMN03774768 | KT186967 - KT186976 | 10113 | Bow Street Bell | [23, 24, 25] | 1b | Euthanased | Organs | 09/03/2004 | Vaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Vredenheim-E040062 | 1/E.cab-tc/ZAF/04/Vdm-E040062 | SAMN03774784 | KT186987 - KT186996 | 10978 | Bruin Perd | [23, 24, 25] | 1b | Died | Blood | 15/03/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Troughend-E040064 | 1/E.cab-tc/ZAF/04/Tgd-E040064 | SAMN03774783 | KT186997 - KT187006 | 11652 | SA Saddlehorse 2 | [23, 24, 25] | 1b | Died | Organs | 17/03/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Vredenheim-E040065 | 1/E.cab-tc/ZAF/04/Vdm-E040065 | SAMN03774785 | KT187007 - KT187016 | 5823 | Bruin Perd | [23, 24, 25] | 1b | Died | Organs | 17/03/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Avontuur-E040066 | 1/E.cab-tc/ZAF/04/Avt-E040066 | SAMN03774769 | KT187017 - KT187026 | 4128 | Special Edition | [23, 24, 25] | 1b | Died | Organs | 18/03/2004 | Vaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Avontuur-E040081 | 1/E.cab-tc/ZAF/04/Avt-E040081 | SAMN03774770 | KT187037 - KT187046 | 15663 | 2003Stars and Stripes | [23, 24, 25] | 1b | Died | Organs | 24/03/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Kalbaskraal-E040086 | 1/E.cab-tc/ZAF/04/Kbk-E040086 | SAMN03774776 | KT187047 - KT187056 | 7654 | Amber | [23, 24, 25] | 1b | Died | Organs | 29/03/2004 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2004/Avontuur-E040061 | 1/E.cab-tc/ZAF/04/Avt-E040061 | SAMN03780052 | KT186977 - KT186986 | 11336 | Special Edition | [23, 24, 25] | 1c | Died | Blood | 16/03/2004 | Vaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2011/Marme-E110143_1 | 1/E.cab-tc/ZAF/11/Mre-E110143_1 | SAMN03765259 | KT070447 - KT070456 | 3017 | Ruben | [26] | 2 | Died | Organs | 27/02/2011 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2011/Marme-E110180_WC165 | 1/E.cab-tc/ZAF/11/Mre-E110180_WC165 | SAMN03765262 | KT070477 - KT070486 | 3567 | Trigger | [26] | 2 | Died | Organs | 06/03/2011 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2011/Marme-E110180_WC61 | 1/E.cab-tc/ZAF/11/Mre-E110180_WC61 | SAMN03765261 | KT070507 - KT070516 | 5300 | Jordan | [26] | 2 | Subclinical | Blood | 06/03/2011 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2011/Marme-E110180_WC44 | 1/E.cab-tc/ZAF/11/Mre-E110180_WC44 | SAMN03765260 | KT070487 - KT070496 | 2970 | Smokey | [26] | 2 | Recovered | Blood | 06/03/2011 | Vaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2011/Marme-E110418_1 | 1/E.cab-tc/ZAF/11/Mre-E110418_1 | SAMN03765264 | KT070497 - KT070506 | 6806 | Moon | [26] | 2 | Died | Organs | 22/03/2011 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2011/Marme-E110411_1 | 1/E.cab-tc/ZAF/11/Mre-E110411_1 | SAMN03765263 | KT070457 - KT070466 | 4566 | Dusty | [26] | 2 | Died | Organs | 26/03/2011 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2011/Marme-E110674_3 | 1/E.cab-tc/ZAF/11/Mre-E110674_3 | SAMN03765265 | KT070467 - KT070476 | 6847 | Robin | [26] | 2 | Recovered | Blood | 18/04/2011 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Saron-E140526_WC00502 | 1/E.cab-tc/ZAF/14/Srn-E140526_WC00502 | SAMN03769430 | KT187187 - KT187196 | 10105 | McGuiver | TS | 3a | Subclinical | Blood | 24/03/2014 | Vaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Saron-E140526_WC00493 | 1/E.cab-tc/ZAF/14/Srn-E140526_WC00493 | SAMN03769429 | KT187177 - KT187186 | 13406 | Milky | TS | 3a | Subclinical | Blood | 24/03/2014 | Vaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Saron-E140526_WC00481 | 1/E.cab-tc/ZAF/14/Srn-E140526_WC00481 | SAMN03769427 | KT187137 - KT187146 | 7881 | Ballaka | TS | 3a | Subclinical | Blood | 24/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Saron-E140526_WC00488 | 1/E.cab-tc/ZAF/14/Srn-E140526_WC00488 | SAMN03769428 | KT187157 - KT187166 | 18131 | King | TS | 3a | Subclinical | Blood | 24/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Saron-E140526_WC00491 | 1/E.cab-tc/ZAF/14/Srn-E140526_WC00491 | SAMN03774780 | KT187167 - KT187176 | 8216 | Fire | TS | 3a | Recovered | Blood | 24/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Saron-E140526_WC00482 | 1/E.cab-tc/ZAF/14/Srn-E140526_WC00482 | SAMN03774779 | KT187147 - KT187156 | 8624 | Raily | TS | 3a | Died | Blood | 24/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Porterville-E140536_WC00507 | 1/E.cab-tc/ZAF/14/Ptv-E140536_WC00507 | SAMN03769425 | KT187207 - KT187216 | 12686 | Jahan Zahmar | TS | 3a | Recovered | Blood | 27/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Porterville-E140536_WC00508 | 1/E.cab-tc/ZAF/14/Ptv-E140536_WC00508 | SAMN03769426 | KT187217 - KT187226 | 10474 | Jahan Dajinah | TS | 3a | Recovered | Blood | 27/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Porterville-E140536_WC00506 | 1/E.cab-tc/ZAF/14/Ptv-E140536_WC00506 | SAMN03769424 | KT187197 - KT187206 | 14902 | Jahan Nasibah | TS | 3a | Recovered | Blood | 27/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Porterville-E140485_WC00522 | 1/E.cab-tc/ZAF/14/Ptv-E140485_WC00522 | SAMN03769419 | KT187077 - KT187086 | 15126 | Jahan Faraj | TS | 3a | Recovered | Blood | 31/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Porterville-E140485_WC00544 | 1/E.cab-tc/ZAF/14/Ptv-E140485_WC00544 | SAMN03769422 | KT187117 - KT187126 | 9638 | Jahan Dhia | TS | 3a | Subclinical | Blood | 31/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Porterville-E140485_WC00528 | 1/E.cab-tc/ZAF/14/Ptv-E140485_WC00528 | SAMN03769420 | KT187097 - KT187106 | 10108 | Jahan Jihad | TS | 3a | Recovered | Blood | 31/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Porterville-E140485_WC00533 | 1/E.cab-tc/ZAF/14/Ptv-E140485_WC00533 | SAMN03769421 | KT187107 - KT187116 | 8122 | Jahan Al Rabiah | TS | 3a | Recovered | Blood | 31/03/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Porterville-E140485_WC00555 | 1/E.cab-tc/ZAF/14/Ptv-E140485_WC00555 | SAMN03769423 | KT187127 - KT187136 | 5588 | el-Jahrousse Mia | TS | 3a | Recovered | Blood | 31/03/2014 | Vaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Robertson-E140702_RB00008 | 1/E.cab-tc/ZAF/14/Rbn-E140702_RB00008 | SAMN03774777 | KT187087 - KT187096 | 6356 | 2013Hersonet | TS | 3b | Recovered | Blood | 25/04/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/E.caballus-tc/ZAF/2014/Robertson-E140816_RB00221 | 1/E.cab-tc/ZAF/14/Rbn-E140816_RB00221 | SAMN03774778 | KT187227 - KT187236 | 6721 | 2013Tale Of The Glacier | TS | 3b | Subclinical | Blood | 08/05/2014 | Unvaccinated |
| African horse sickness virus 1 strain 1/Labstr/ZAF/1998/OBP-116 | 1/Lab/ZAF/98/OBP-116 | SAMN03764401 | KT030330 - KT030339 | 7547 | OBP AHSV1 LAV, Batch 116 | [41] | | | | | |
| African horse sickness virus 2 strain 2/Labstr/ZAF/1998/OBP-252.1 | 2/Lab/ZAF/98/OBP-252.1 | SAMN04038462 | KT715601 - KT715610 | 29050 | OBP AHSV2 LAV, Batch 252.1 | [42] | | | | | |
| African horse sickness virus 3 strain 3/Labstr/ZAF/1998/OBP-116 | 3/Lab/ZAF/98/OBP-116 | SAMN03764402 | KT030340 - KT030349 | 11756 | OBP AHSV3 LAV, Batch 116 | [41] | | | | | |
| African horse sickness virus 4 strain 4/Labstr/ZAF/1998/OBP-116 | 4/Lab/ZAF/98/OBP-116 | SAMN03764403 | KT030350 - KT030359 | 6977 | OBP AHSV4 LAV, Batch 116 | [41] | | | | | |
| African horse sickness virus 6 strain 6/Labstr/ZAF/1998/OBP-252.1 | 6/Lab/ZAF/98/OBP-252.1 | SAMN04038463 | KT715611 - KT715620 | 35724 | OBP AHSV6 LAV, Batch 252.1 | [42] | | | | | |
| African horse sickness virus 7 strain 7/Labstr/ZAF/1998/OBP-252.1 | 7/Lab/ZAF/98/OBP-252.1 | SAMN04038464 | KT715621 - KT715630 | 137497 | OBP AHSV7 LAV, Batch 252.1 | [42] | | | | | |
| African horse sickness virus 8 strain 8/Labstr/ZAF/1998/OBP-252.1 | 8/Lab/ZAF/98/OBP-252.1 | SAMN04038465 | KT715631 - KT715640 | 120396 | OBP AHSV8 LAV, Batch 252.1 | [42] | | | | | |
| African horse sickness virus 1 strain 1/Labstr/ZAF/1962/OVI-HS29/62 | 1/Lab/ZAF/62/OVI-HS29/62 | SAMN03765190 | KT030570 - KT030579 | 8401 | ARC-OVI OIE Laboratory Reference Strain AHSV1 | [44], TS | | | | | |
| African horse sickness virus 2 strain 2/Labstr/ZAF/1961/OVI-HS82/61 | 2/Lab/ZAF/61/OVI-HS82/61 | SAMN03765191 | KT030580 - KT030589 | 7698 | ARC-OVI OIE Laboratory Reference Strain AHSV2 | [44], TS | | | | | |
| African horse sickness virus 3 strain 3/Labstr/ZAF/1963/OVI-HS13/63 | 3/Lab/ZAF/63/OVI-HS13/63 | SAMN03765192 | KT030590 - KT030599 | 5281 | ARC-OVI OIE Laboratory Reference Strain AHSV3 | [44], TS | | | | | |
| African horse sickness virus 4 strain 4/Labstr/ZWE/1962/OVI-HS32/62 | 4/Lab/ZWE/62/OVI-HS32/62 | SAMN03765193 | KT030600 - KT030609 | 11579 | ARC-OVI OIE Laboratory Reference Strain AHSV4 | [44], TS | | | | | |
| African horse sickness virus 5 strain 5/Labstr/ZAF/1962/OVI-HS30/62 | 5/Lab/ZAF/62/OVI-HS30/62 | SAMN03765194 | KT030610 - KT030619 | 8637 | ARC-OVI OIE Laboratory Reference Strain AHSV5 | [44], TS | | | | | |
| African horse sickness virus 6 strain 6/Labstr/ZAF/1963/OVI-HS39/63 | 6/Lab/ZAF/63/OVI-HS39/63 | SAMN03765195 | KT030620 - KT030629 | 12422 | ARC-OVI OIE Laboratory Reference Strain AHSV6 | [44], TS | | | | | |
| African horse sickness virus 7 strain 7/Labstr/ZAF/1962/OVI-HS31/62 | 7/Lab/ZAF/62/OVI-HS31/62 | SAMN03765197 | KT030640 - KT030649 | 9632 | ARC-OVI OIE Laboratory Reference Strain AHSV7 | [44], TS | | | | | |
| African horse sickness virus 8 strain 8/Labstr/KEN/1962/OVI-HS10/62 | 8/Lab/KEN/62/OVI-HS10/62 | SAMN03765198 | KT030650 - KT030659 | 9208 | ARC-OVI OIE Laboratory Reference Strain AHSV8 | [44], TS | | | | | |
| African horse sickness virus 9 strain 9/Labstr/TCD/1961/OVI-HS90/61 | 9/Lab/TCD/61/OVI-HS90/61 | SAMN03765199 | KT030660 - KT030669 | 4957 | ARC-OVI OIE Laboratory Reference Strain AHSV9 | [44], TS | | | | | |

[a] Strain Name is formatted as follows: <virus name> strain <type>/<isolation host-suffix>/<country of sampling>/<year of sampling>/<variant designation>-<isolate designation> where <virus name> is the taxonomic name of the virus, <type> is the type of the virus strain, <isolation host-suffix> the first letter of genus name.full name of species from which the samples were collected and the suffix -tc indicates the strain was propagated on tissue culture, <country of sampling> is the ISO 3166-1 alpha-3 code for the country of sampling, <year of sampling> is the 4 digit year in which the sample was collected, <variant designation> is the name of the region in which the sample was collected and <isolate designation> is the laboratory code given to the specific sample.

[b] Abbreviated Strain Name is formatted as follows: <type>/<isolation host-suffix>/<country of sampling>/<year of sampling>/<variant designation>-<isolate designation> where <type> is the type of the virus strain, <isolation host-suffix> four-letter format comprising first letter of genus name.first three letters of species from which the samples were collected and the suffix -tc indicated that the strain was propagated on tissue culture, <country of sampling> is the ISO 3166-1 alpha-3 code for the country of sampling, <year of sampling> is the 2 digit year in which the sample was collected and <variant designation> 3 letter code for the name of the region in which the sample was collected and <isolate designation> is the laboratory code given to the specific sample.



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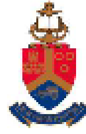
# APPENDIX E



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**ANIMAL USE AND CARE COMMITTEE**
Private Bag X04
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Tel +27 12 529 8434 / Fax +27 12 529 8300
e-mail: aucc@up.ac.za

**Ref: V029-12**

29 May 2012

Prof AJ Guthrie
Equine Research Centre
Faculty of Veterinary Science
( alan.guthrie@up.ac.za )

Dear Prof Guthrie

**V029-12 : Characterization of the dynamics of African horse sickness viraemia in horses vaccinated with commercial modified live virus vaccine using real time polymerase chain reaction assays *(C Weyer)***

The application for ethical approval, dated 15 May 2012 was approved by the Animal Use and Committee at its meeting held on 28 May 2012.

Kind regards

**Elmarie Mostert**

**AUCC Coordinator**

Copy Dr Weyer



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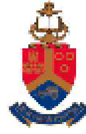
# APPENDIX F



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**Ref: V030-12**

29 May 2012

Prof AJ Guthrie
Equine Research Centre
Faculty of Veterinary Science
( alan.guthrie@up.ac.za )

Dear Prof Guthrie

**V030-12 : Development and initial characterization of sterotype specific RT-PCR assays for African horse sickness *(C Weyer)***

The application for ethical approval, dated 15 May 2012 was approved by the Animal Use and Committee at its meeting held on 28 May 2012.

Kind regards

**Elmarie Mostert**

**AUCC Coordinator**

Copy Dr Weyer



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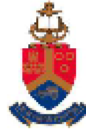
# APPENDIX G



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**Ref: V031-12**

29 May 2012

Prof AJ Guthrie
Equine Research Centre
Faculty of Veterinary Science
( alan.guthrie@up.ac.za )

Dear Prof Guthrie

**V031-12 : A survey of foals in the AHS surveillance zone followig the 2011 Mamre outbreak**

***(C Weyer)***

The application for ethical approval, dated 15 May 2012 was approved by the Animal Use and Committee at its meeting held on 28 May 2012.

Kind regards

**Elmarie Mostert**

**AUCC Coordinator**

Copy Dr Weyer



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