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Short communication

Using a new serotype-specific Polymerase Chain Reaction (PCR) and sequencing to differentiate between field and vaccine-derived African Horse Sickness viruses submitted in 2016/2017

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ABSTRACT

The outer capsid viral protein 2 (VP2) of African horse sickness virus, encoded by the most variable genome segment 2 (Seg-2), is the primary target for AHSV-specific neutralising antibodies and thus determines the virus serotype. Full length segment 2 sequences from more than 100 AHSVs isolated over the last 80 years were compared and single nucleotide polymorphisms (SNPs) identified between the reference strains and recent field viruses. Regions unique to each individual serotype were identified and primers designed to differentially amplify each of the nine serotypes. The sequences of resulting amplicons contained a significant amount of SNPs to discriminate between field viruses and reference strains or live attenuated viruses. The new serotype specific RT-PCR were subsequently used to determine the prevalence of different AHSV serotypes associated with samples submitted to the Agricultural Research Council - Onderstepoort Veterinary Research Institute during the 2016 / 2017 season. Subsequent sequencing of the PCR products were used to determine if the infections were caused by field or vaccine-derived strains. The serotypes of 70 AHSV positive diagnostic samples submitted to the ARC-OVR were determined. Serotypes 2 and 6 were the most prevalent, while Serotype 1 was the only serotype where sequences identical to the ALV or reference strains were detected in field samples. Based on this study, the incidence of vaccine-derived AHS infections submitted from southern Africa were low. This serotype-specific RT-PCR and sequencing assay could assist with the surveillance and control of equines movement nationally and internationally. It could also provide valuable scientific guidance on the policies and guidelines regulating vaccination and trade of equines in South Africa.

The outer capsid viral protein 2 (VP2) of African horse sickness virus, encoded by the most variable genome segment 2 (Seg-2), is the primary target for AHSV-specific neutralising antibodies and thus determines the virus serotype. Full length segment 2 sequences from more than 100 AHSVs isolated over the last 80 years were compared and single nucleotide polymorphisms (SNPs) identified between the reference strains and recent field viruses. Regions unique to each individual serotype were identified and primers designed to differentially amplify each of the nine serotypes. The sequences of resulting amplicons contained a significant amount of SNPs to discriminate between field viruses and reference strains or live attenuated viruses. The new serotype specific RT-PCR were subsequently used to determine the prevalence of different AHSV serotypes associated with samples submitted to the Agricultural Research Council - Onderstepoort Veterinary Research Institute during the 2016 / 2017 season. Subsequent sequencing of the PCR products were used to determine if the infections were caused by field or vaccine-derived strains. The serotypes of 70 AHSV positive diagnostic samples submitted to the ARC-OVR were determined. Serotypes 2 and 6 were the most prevalent, while Serotype 1 was the only serotype where sequences identical to the ALV or reference strains were detected in field samples. Based on this study, the incidence of vaccine-derived AHS infections submitted from southern Africa were low. This serotype-specific RT-PCR and sequencing assay could assist with the surveillance and control of equines movement nationally and internationally. It could also provide valuable scientific guidance on the policies and guidelines regulating vaccination and trade of equines in South Africa.

African horse sickness virus (AHSV) causes a non-contagious infection affecting equid species (Coetzer and Guthrie, 2004). The virus is transmitted to susceptible animals by female biting midges of the genus *Culicoides,* following a blood meal containing high concentrations of infectious virus (Du Toit, 1944). This arbovirus belongs to the genus

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Orbivirus in the family *Reoviridae* (Mertens et al., 2005). It is a non-enveloped virus and contains ten double-stranded RNA genome segments encoding seven structural and four non-structural proteins (Bremer, 1976). The outer protein layer of the double capsid virus is composed of trimers of VP2, which are responsible for the serotype specific antigenicity of the virus (Burrage et al., 1993; Martinez-Torrecuadrada et al., 1994). Nine distinct viral serotypes have been identified, each with a serotype-specific VP2 protein, which is significantly different between the serotypes (McIntosh, 1958; Howell, 1962). This protein is encoded by the highly variable genome segment 2 (Potgieter et al., 2003), which is the target for numerous serotype-specific reverse transcription PCR assays (Sailleau et al., 2000; Koekemoer, 2008; Bachanek-Bankowska et al., 2014; Weyer et al., 2015).

African horse sickness is endemic to South Africa, where seasonal outbreaks are officially recorded from the 1 st of September to 31 August of the following year, so as to include all cases from a single summer season (Department of Agriculture, Forestry and Fisheries, RSA). All nine serotypes of AHSV have been reported in South Africa and since 2006 all nine provinces have reported cases annually, with the exception of the Free State in 2007, the Northern Cape in 2012 and the Western Cape in 2010 and 2012 (Department of Agriculture, Forestry and Fisheries, RSA). Annual vaccination is compulsory in South Africa, with the exception of the Free and Surveillance Zones in the Western Cape Province. Vaccination should occur within the period of low vector activity between 1 June and 31 October. The African Horse Sickness Vaccine from Onderstepoort Biological Products (Reg No, G0116, Act 36 of 1947) is currently the only registered AHS vaccine in terms of the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947, in South Africa (Act No. 36 of 1947). This vaccine consists of a polyvalent mixture of attenuated live viruses (ALVs) and was developed in the 1970's by the repetitive selection of large plaque variants in cell culture until an attenuated strain was obtained (Erasmus, 1978). The AHSV reference strains as described by Potgieter et al. (2015), were initially used to generate the cell-culture attenuated vaccine strains and genetic differences between the reference strains and ALVs were described by Guthrie et al. (2015a), and Guthrie et al. (2015b). This AHS vaccine contains seven of the nine ASHV serotypes formulated as a trivalent and tetravalent application, administered 21 days apart. The serotype combinations present in each of these vaccine applications, were selected based on cross-neutralisation results in order to minimise the interference between the serotypes. Bottle I, contains serotype 1, 3 and 4, while Bottle II is composed of serotypes 2, 6, 7 and 8 (von Teichman and Smith, 2008). A disadvantage of the current vaccine is that it is not possible to discriminate between vaccinated and naturally infected animals. This problem is exacerbated by possible reassortment with field viruses and reversion to virulence following vaccine-associated infection (Weyer et al., 2016).

The purpose of this study was firstly to design a serotype-specific RT-PCR and determine the sequences of the resulting amplicons. Based on these sequences a distinction between circulating field viruses and either reference strains or vaccine ALVs could be made. The second aim was to apply this assay to estimate the prevalence of individual serotypes during one season and observe the frequency of vaccine-derived AHS outbreaks.

Full length sequences of segment 2 from 179 AHSVs were obtained from GenBank at the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov). These included the nine reference strains (Potgieter et al., 2015), the seven ALVs (Guthrie et al., 2015a,b) and 128 field viruses isolated from the 1930's to 2014. Alignments of segment 2 were constructed using CLC Genomics Workbench (Qiagen, Aarhus: www.clcbio.com). Multiple alignments were performed and regions unique to each individual serotype were identified. Single nucleotide polymorphisms (SNPs) were identified amongst the field isolates, the reference and ALV for each individual serotype (Table 1). Nine unique sets of primers were designed, each set amplifying only a

Table 1
Sequences of the nine pairs of serotype specific primers, their hybridization position on segment 2 and the resulting amplicon size. The number of single nucleotide polymorphisms between the ALV, reference viruses and field samples within the amplified region and over the complete segment 2.

Serotype	Forward Primer		Reverse Primer		Forward Primer	Reverse Primer	Size amplicon	# SNPs / PCR amplicon	# SNPs / Segment 2	# SNPs between reference and ALV ** (# within PCR amplicon)
	Name	Sequence	Name	Sequence	Annealing position on Seg 2 (bp)		(bp)			
AHSV-1	AHSV-1-F	GAATGCTCGATATGAGGTTTC	AHSV-1-R	TCACTCACGCTTTGTTTATGTTCA	666	1172	507	10	33	3 (1)
AHSV-2	AHSV-2-F	GTTGGAAGTATGCCTAAAATGGA	AHSV-2-R	CCATACGGCACATCTGTTCG	1007	1261	255	11	35	1 (0)
AHSV-3	AHSV-3-F	CTAGACGGCTTTGCGC	AHSV-3-R	TCATCAACCTCTTTACTCCATTC	1463	1685	223	14	104	4 (1)
AHSV-4	AHSV-4-F	GAGTTTCAAATTTGAGGGGTT	AHSV-4-R	GAATTAAAGTATCCATCGAACCC	2659	3117	459	8	31	3 (0)
AHSV-5	AHSV-5-F	GGATTGGAGCGTTACAGG	AHSV-5-R	CAATGTATTTAGGGGTCCGAC	516	800	285			
AHSV-6	AHSV-6-F	CGTACTGGTATCGTGTTGAG	AHSV-6-R	TAAGTCTTGCTGCTGCGAA	1920	2320	401	11	36	2 (1)
AHSV-7	AHSV-7-F	CATTTCAATGGGTGGATGGAT	AHSV-7-R	GGATCTCCATCTTCTCCATTACAC	2660	2953	294	*		12 and 674 deletion (1)
AHSV-8	AHSV-8-F	CAAGTGGATACAGGGGATGC	AHSV-8-R	CCCAATTTTGAAGGGTCCC	1006	1290	285	7	58	2 (1)
AHSV-9	AHSV-9-F	GATGTTGGATAATGTCGTGTACTG	AHSV-9-R	GATCGAGCATCCTAGTACAGAAC	2180	2422	243			

*No SNPs determined but a 674 bp deletion detected.
**According to Guthrie et al. (2015a), and (2015b).

Table 2
African horse sickness viruses used during the design and evaluation of the serotype-specific RT-PCR, as well as its implementation during the 2016–2017 season.

	AHSV-1	AHSV-2	AHSV-3	AHSV-4	AHSV-5	AHSV-6	AHSV-7	AHSV-8	AHSV-9	Total
Full VP2 (NGS)	**56**	**11**	**14**	**22**	**13**	**18**	**12**	**18**	**15**	**179**
Samples used during the validation of the serotype-specific RT-PCR										
Vaccine	1 (OBP)	1 (OBP)	1 (OBP)	1 (OBP)	0	1 (OBP)	1 (OBP)	1 (OBP)	0	7
Proficiency Panel	1 (Ref)	20 (1 Ref)	3 (2 Ref)	0	1	2	5	9 (1 Ref)	7	48
Historic samples	6	3	2	1	2	1	1	2	0	18
Field (2014 / 2015)	1 (ALV)					5	2	7		15
Field (2015 / 2016)	1 (ALV)	2	4 (Part of a vaccine trial)	1		1	2	14	7	32
Samples tested	**10**	**26**	**10**	**3**	**3**	**10**	**11**	**33**	**14**	**120**
Positive AHS samples, submitted to ARC-OVI from September 2016 to July 2017										
Field (2016 / 2017)	8 (1 Ref)	22	–	1	–	22	4	9	4	70

OBP: Indicate the use of commercial vaccine as template for PCR and sequencing.
Ref: Sequence identical to the reference strain was obtained.
ALV: Sequence identical to the ALV strain was obtained.

specific serotype. Specific annealing to an individual serotype was verified *in silico* using CLC Genomics Workbench (Table 1). The predicted amplicons ranged from 222 to 506bp and their sequences contained more than 10% of the SNPs between field viruses and ALVs over the complete segment 2 (Table 1). Six of the serotype-specific amplicons had between 7 and 14 SNPs, while an additional primer set was designed to differentiate between field viruses and the ALV of serotype 7, since the latter contains a 670bp deletion in segment 2 when compared to the reference strain of serotype 7 (Guthrie et al., 2015b). The nine different primer combinations were evaluated using dsRNA from the nine reference strains as template as well as a cloned segment 2 from each serotype as positive control. This was performed using SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* DNA polymerase (Invitrogen, Thermofisher), in a 20 µl reaction with 0.25 µM of each primer, at an annealing temperature of 58 °C, for 45 cycles. A 2 µl aliquot of the resulting amplicons were analysed on a 1% agarose gel. A sample was assigned to a specific serotype if an amplicon was present in the RT-PCR with the corresponding primer set (Supplementary Fig. 1). The assays were used to test 120 samples (Table 2). These included the commercial vaccine available from Onderstepoort Biological Products, 48 samples from a panel which included five reference strains and 18 viruses isolated from diagnostic samples submitted to the ARC-OVI and stored as lypholized virus. The serotype of each of the 120 samples had been determined by either RT-PCR for the 48 panel samples (Bachanek-Bankowska et al., 2014) or serologically for the lypholized viruses. Fifteen AHS positive diagnostic samples were analysed from the 2014 / 2015 and 32 from the 2015 / 2016 season (Table 2). Since the assay could be used to distinguish between natural and vaccine-associated infection based on the sequence of the amplicons, the assay was also used to determine the prevalence of AHSV serotypes and vaccine-associated infections during the 2016 / 2017 season. Seventy AHS positive samples were serotyped and their sequences were used to estimate the frequency of vaccine-derived AHS outbreaks (Table 2).

The 2016 / 2017 season is defined from September 2016 to July 2017 and samples from South Africa, Swaziland, Mozambique, Zimbabwe and Malawi were included in the analysis. The majority of the positive samples were submitted from either Gauteng or North West province (22.6% each), followed by the Northern Cape (17%), Mpumalanga (12.9%), Free State (9.7%) and Limpopo (8%) (Fig. 1). No positive samples were received from either the Western Cape or the Eastern Cape. This is not a reflection of the prevalence or distribution of AHS in South Africa, since only samples submitted to the ARC-OVI were included in this study. Total nucleic acid was extracted from either blood in ethylene-diamine-tetra-acetic acid (EDTA) vacutainer tubes or tissue samples, submitted to the ARC-OVI for AHSV testing, using the MagNA Pure 96 (Roche, Molecular Systems Inc. Germany). The presence of AHSV nucleic acid was determined by either hemi-nested RT-PCR assay (Bremer et al., 1998) or real-time RT-PCR (Agüero et al., 2008). Only nucleic acid of samples that tested positive for AHSV were subjected to serotype-specific PCR. The amplicons generated during the aforementioned serotype-specific RT-PCR, were submitted for sequencing to Inqaba Biotechnical Industries Pty Ltd (Pretoria, South Africa). Sequencing reactions using the forward primer of the specific serotype were performed with Bigdye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on a 3500 Genetic Analyzer (ThermoFisher Scientific). Sequence alignments were constructed for each individual serotype, containing both data obtained from GenBank as well as the data generated from serotype-specific RT-PCR amplicons using Clustal W in the CLC Genomics Workbench. Maximum-likelihood trees were constructed using GTR + T4 with 1000 replicates bootstrapping in Mega 6 (Tamura et al., 2013).

Serotype 2 and 6 were the most prevalent with 32% of the samples identified as either of these two serotypes (Table 2). This was followed by Serotype 8 (12.7%) and serotype 1 (11%), while serotypes 9 (5.6%), 7 (4%) and 4 (1.4%) were all less than 10% of the total. No serotypes 3 or 5 were detected in this season or the previous two seasons. The only serotype 3 samples that were submitted were part of a vaccine trail in 2015 / 2016 and is thus not representative of the serotype prevalence, but were included during the evaluation of the serotype-specific PCR assay. These observations are similar to data published on the prevalence of AHS in Onderstpoort in 1995 / 1996, where serotypes 2 and 4 had the highest prevalence, followed by serotypes 1, 6 and 9 (Bremer et al., 2000). Gordon et al. (2017) determined serotype 7 (33%) to be dominant in Zimbabwe, followed by serotype 2 (26%) and serotype 4 and 8 at 16% each. This is indicative of AHS in endemic areas, where different serotypes occur simultaneously with one being dominant during one season followed by another in the following (Coetzer and Erasmus, 1994).

Since 2010, Gauteng province had the highest number of AHS cases reported and together with North West, Mpumalanga and the Northern Cape accounts for more than 70% of the annual reported cases (Department of Agriculture, Fisheries and Forestry, Republic of South Africa, 2019). These four provinces were not only accredited with the most samples submitted to the ARC-OVI, but also the highest number of serotypes (Fig. 1). Four of five different serotypes were identified in each of these provinces and the majority of these samples were either related or identical. This implies significant amount of virus migration through these neighbouring provinces, probably associated with horse or game (notable zebra) movement. Concerns have been expressed in the past that the restocking of various areas with zebra, which can serve as asymptomatic carriers of AHSV, has created conditions that could allow for ongoing circulation and hence further sources of outbreaks amongst horses (Barnard, 1998). The data presented here suggests that the viruses were circulating over successive seasons, rather than being new introductions as was previously speculated (Coetzer and Erasmus, 1994).

Based on the sequences obtained for the regions under investigation,

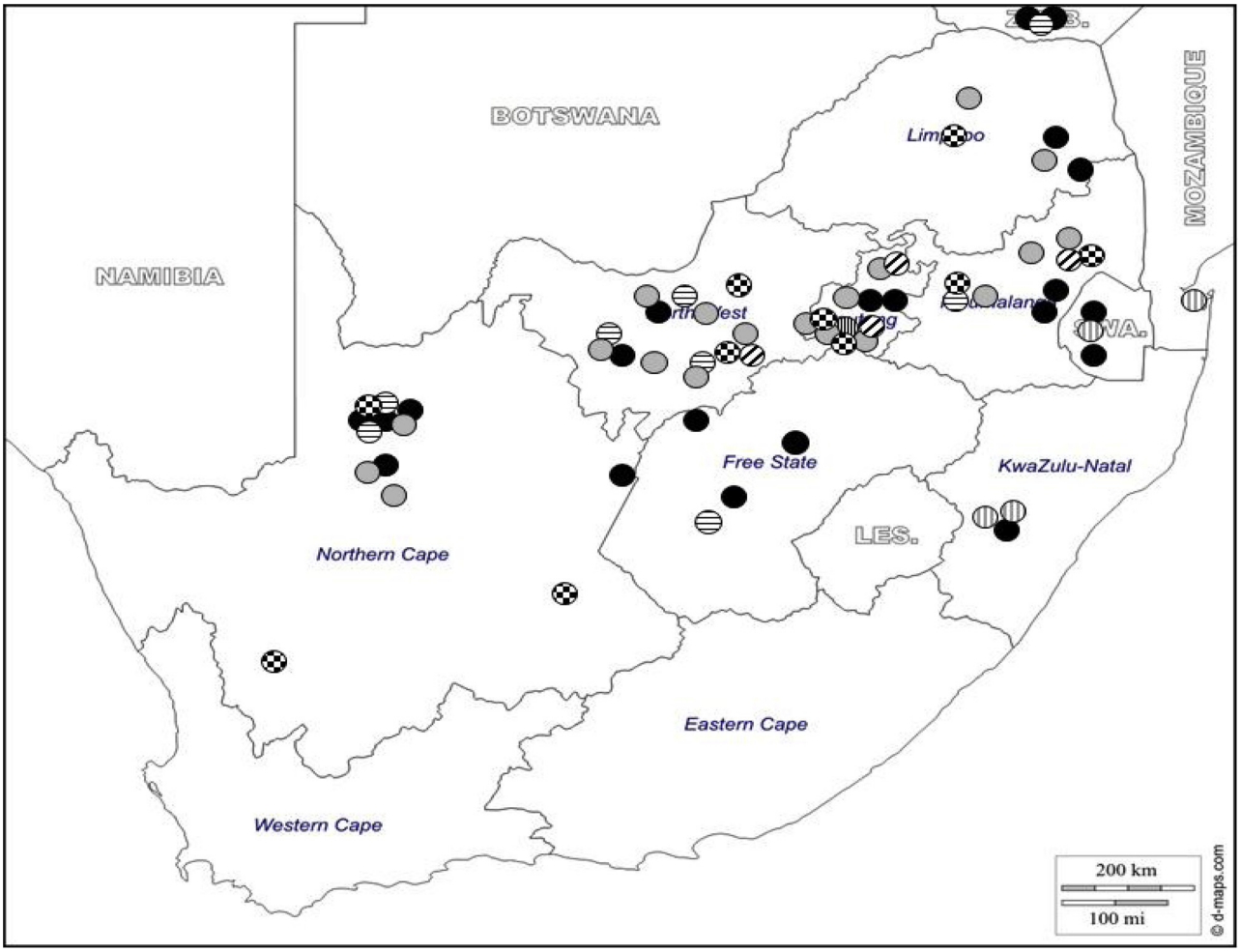

Fig. 1. Prevalence distribution of AHSV serotypes in South Africa during the 2016 / 2017 season. The different serotypes are indicated with circles as described next to the map. The Map was generated using Geocortex viewer for Html5 using an image from d-maps.com.

the rate of substitution was low and the majority of the substitutions were non-synonymous. This is in accordance with observations from other arboviruses, where the obligate replication in alternative host and vector inhibits rapid virus evolution (Carpi et al., 2010). Despite this, a clear phylogenetic distinction between the reference, ALV strains and the field isolates were observed for all the serotypes (Fig. 2).

Serotype 1 was the only serotype where sequences identical to the ALV or reference strains were detected in field samples (Fig. 2A). Sample PS2367 was submitted from the Northern Cape in 2016 / 2017 and its sequence was identical to the reference strain (KP939376_AHSV-1_29_62). Within the amplified region of interest, the reference strain differs from the ALV based on one nucleotide at position 1071 of segment 2 (Guthrie et al., 2015b; Table 1). Weyer et al., 2016 described this SNP to result in an amino acid change at position 357 of Asparagine (N) to Lysine (K). Two samples (PS2221 and PS2187) were both submitted shortly after vaccination in 2015/ 2016. In both cases the horses only displayed fever and the resulting amplicons had sequences identical to the ALV (KT030331_AHSV-1 /labstr/ZAF/1998/OBP-116), indicating that the symptoms were vaccine derived. These samples from serotype 1 were the only evidence of vaccine-derived outbreak and based on the phylogenetic comparisons between sequences of the reference / ALV strains and the field samples submitted to the ARC-OVI, no evidence for the circulation of vaccine-derived strains were detected in any of the remaining serotypes (Fig. 2).

Serotype 2 had the highest prevalence, as well as the highest diversity with nine distinct clusters identified when analysing samples submitted during the 2016 / 2017 season (Fig. 2B). In contrast to serotype 2, serotype 6 had a high prevalence, but lower genetic diversity. All the South African samples grouped into seven clusters with a high percentage sequence identity between the different clusters (Fig. 2C). Sample PS2538 was submitted from Malawi and it shared a common recent ancestor with reference strain AHSV-6_39-63, isolated in 1963 from South Africa (Fig. 2C). The latter is the only other evidence for the likely circulation of a vaccine-related strain, since it shares between 97.52 and 97.83 percent sequence identity with the reference (KP939816_AHSV-6_2-1975) and ALV (KT715612_AHSV-6 /Labstr/ZAF/1998/OBP-252.1) strains.

In conclusion this study has shown the successful use of a newly developed serotype-specific RT-PCR with the ability to not only differentiate between AHSV serotypes, but based on the sequencing of the segment 2 amplicons, also discriminate between field viruses and either ALV or reference strains. The serotype RT-PCR indicated that serotype 2 and 6 were the most prevalent during the 2016 / 2017 season. The resulting amplicons were sequenced and used to demonstrate the low frequency of vaccine-derived AHS outbreaks. With the exception of one serotype 1 sample, no other vaccine-derived samples were observed during this season. These findings have provided us with valuable information regarding the prevalence, distribution and circulation of specific AHSV strains in southern Africa, with special emphasis on the low frequency of vaccine-derived viruses. The results suggest that the movement of equids in southern Africa is posing a problem leading to virus migration throughout the provinces. Thus the validation and subsequent implementation of this newly developed serotype-specific RT-PCR assay would contribute greatly to future epidemiological

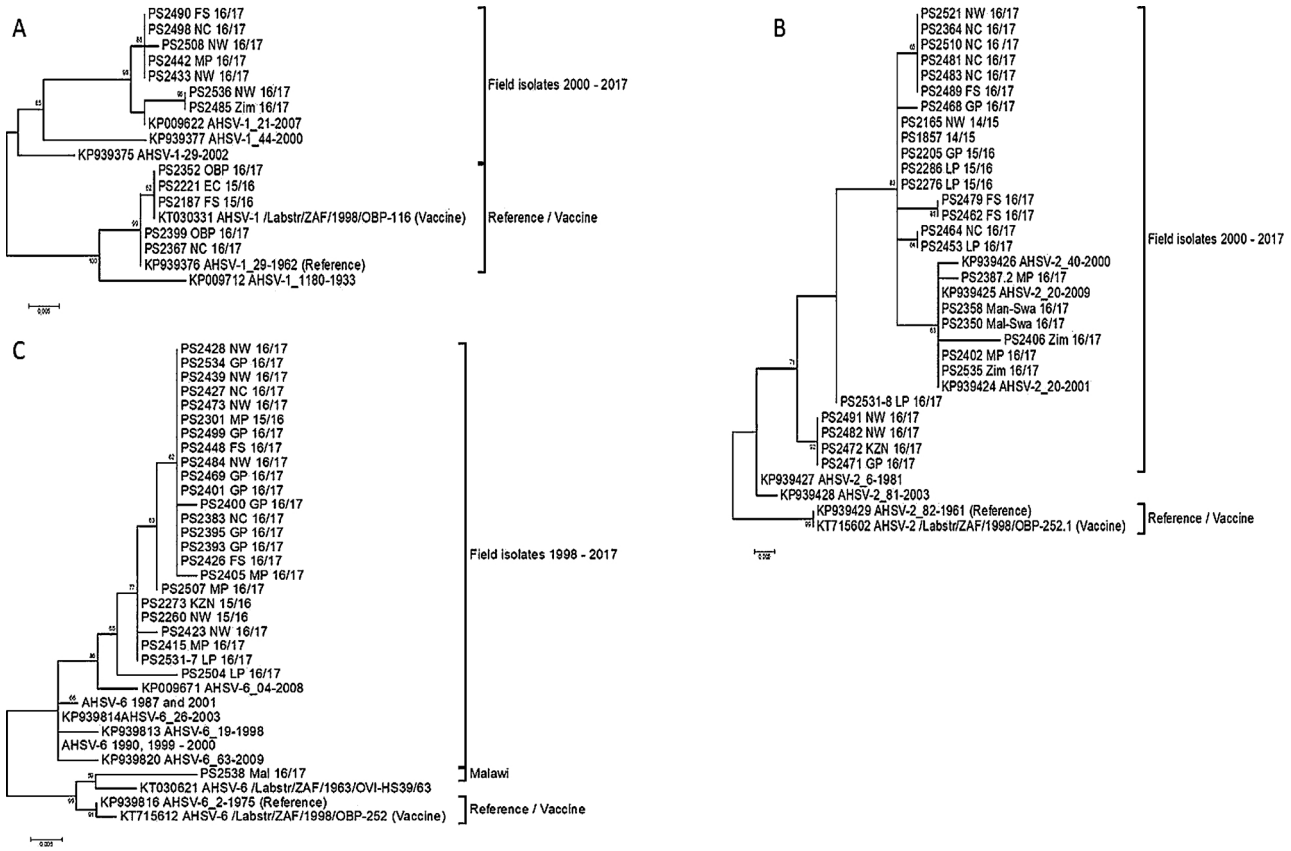

Fig. 2. Maximum likelihood phylogenetic tree for the region of interest from segment 2 of serotype 1 (A), serotype 2 (B) and serotype 6 (C). The origin of a sample is indicated by: GP = Gauteng Province; NW = North West Province; FS = Free State Province; KZN = KwaZulu Natal Province; EC = Eastern Cape Province; NC = Northern Cape Province; LP = Limpopo Province; MP = Mpumalanga Province; Man-Swa = Manzini, Swaziland; Mal-Swa = Malkerns, Swaziland; Zim = Zimbabwe; Mal = Malawi; Nam = Namibia.

studies, control measures and vaccination programmes.

Authors contributions

Dr A. van Schalkwyk: Experimental design, performing the experiments and manuscript writing.

Dr M.L. Ferreira: Performed the experiments.

Dr M. Romito: Contributed AHSV RNA for the validation of the RT-PCR assay and serotyping of the 2016 / 2017 samples.

Significance of work

A new Reverse Transcription – Polymerase Chain Reaction (RT-PCR) assay is described that can differentiate between the nine serotypes of African horse sickness virus (AHSV). A unique set of primers targeting regions in the corresponding genome segment-2 were designed for each serotype. Sequences of the individual amplicons have single nucleotide polymorphisms (SNPs) allowing discrimination between field samples and either reference strains or live attenuated viruses (ALVs). This assay was tested and used to determine the serotype prevalence of AHSV samples submitted during the 2016 / 2017 season as well as to determine the incidence of field samples derived from ALVs. The information was used to study the epidemiology of AHSV in Southern Africa.

Competing interests

The authors declare that they have no financial or personal relationships which may have inappropriately influenced them in writing this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.01.016.

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