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

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Highlights:

- Three triplex AHSV TS RT-qPCR assays that can be applied directly to nucleic acid extracted from blood samples collected from AHSV infected horses are described.
- Multiplexing of the primers and probes for 9 AHSV serotypes increases assay output.
- 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.

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 serotype-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 serotypes, 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 serotypes, 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 serotypes 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.

Keywords: AHS, serotype, RT-qPCR, field diagnosis, equine

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 (Guthrie and Weyer, 2015; MacLachlan and Guthrie, 2010; Coetzer and Guthrie, 2004). Nine serotypes of AHS virus (AHSV) have been described (Howell, 1962; McIntosh, 1958). 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 (Zwart et al., 2015; Roy et al., 1994; Grubman and Lewis, 1992). Genome segment 7 (S7) encodes the inner capsid protein VP7, which is highly conserved among the 9 AHSV serotypes (Quan et al., 2010) and is the basis for several antigen (Laviada et al., 1992; Hamblin et al., 1991), antibody (Maree and Paweska, 2005; Kweon et al., 2003; Wade-Evans et al., 1993; Hamblin et al., 1990), and nucleic-acid based diagnostic assays (Guthrie et al., 2013; Quan et al., 2010;

Fernandez-Pinero et al., 2009; Agüero et al., 2008; Zientara et al., 1995). An outbreak of AHS in a naïve horse population can be devastating, with a cumulative mortality rate of up to 95% (Guthrie and Weyer, 2015; Coetzer and Guthrie, 2004; Mellor and Hamblin, 2004).

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 (Guthrie and Weyer, 2015). 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 serotype of individual virus isolates by virus neutralisation assays (Guthrie and Weyer, 2015; Coetzer and Guthrie, 2004). 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 (Bachanek-Bankowska et al., 2014; Guthrie et al., 2013; Aradaib, 2009; Sailleau et al., 2000; Stone-Marschat et al., 1994). 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 (Quan et al., 2010). 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 (Guthrie et al., 2013; Weyer et al., 2013). Determination of the serotype of AHSV contained in such samples currently requires further evaluation, with additional testing.

Determination of AHSV serotype 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 serotype of the incurring virus. Traditional VI and serotyping was used previously to identify the AHSV-1 serotype involved in an outbreak of AHS in the region in 2004 (Sinclair et al., 2006), 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 (Grewar et al., 2013). 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

neutralization determinants of AHSV (Kanai et al., 2014; Potgieter et al., 2003; Vreede and Huismans, 1994; Burrage et al., 1993) are well suited for accurate, rapid serotype determination. Direct TS RT-PCR assays have been developed (Sailleau et al., 2000); 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 (2008) 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 serotype 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 (Bachanek-Bankowska et al., 2014).

The objective of the current study was to develop and characterize the applicability and practicability (Broeders et al., 2014) 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.

Materials and Methods

Reference Strains of AHSV

South African reference strains of the 9 AHSV serotypes 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 serotypes 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 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 (Guthrie et al., 2013), followed by analysis with the AHSV TS-qPCR assays described below.

Table 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 (Guthrie et al., 2013), blood samples collected from pyrexic horses with signs typical of AHS (Guthrie and Weyer, 2015) 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 (Ref: V030-12). 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 (Guthrie et al., 2013). 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 (Weyer et al., 2013), 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 serotype 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 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 serotypes included in bottle 1 of the

Table 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

Onderstepoort Biological Products AHS vaccine and which have been shown to be serologically distinct on neutralization tests. Triplex 2 included oligonu cleotides for types 2, 5 and 9 and Triplex 3 included oligonucleotides for types 6, 7 and 8. These triplexes were constituted to ensure that serotypes which have been shown to cross-react on neutralization tests were separated (serotypes 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 2.

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 (Guthrie et al., 2013). Group specific RT-qPCR assays were performed immediately after extraction and the plates containing the remaining eluates were stored at -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 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 (Quan et al., 2008). 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 (Guthrie et al., 2013). Cultures showing no CPE after three passages were classified as negative for AHSV.

AHSV serotype determination was done using a plaque inhibition assay (PI) with fish spine beads containing individual type specific antisera, as previously described for Bluetongue virus (Howell et al., 1970) 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 in strict sequence on the surface of the overlay, after which the plates were returned to the incubator. Homologous neutralization was clearly evident around the periphery of a bead by the absence of plaques after 5–6 days incubation.

Results

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

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

	Serotype	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

The numeric values indicate the Cq value for each serotype reference sample. * indicates a Cq value of 40, which is considered negative.

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 (Guthrie et al., 2013). 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 4). Of these, 156 samples were VI positive with virus serotype determined by PI. All 9 serotypes of AHSV were isolated from

these 156 samples, and there was concordance between the serotype 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 Table S1.

Table 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.

Serotype	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 supplementary material Figure S1. AHSV serotype 8 (AHSV-8) was the most common serotype identified amongst field strains of AHSV tested in both 2011 and 2012, with some 30% of all positive samples containing this serotype. All 9 AHSV serotypes were identified among samples from southern Africa evaluated in 2011, whereas neither AHSV-1 nor AHSV-9 was identified in 2012. AHSV serotype was also determined for 7 VI negative but AHSV RT-qPCR positive archived equine blood samples collected in a previous study (Weyer et al., 2013). Of the 7 samples tested, 3 samples tested positive for serotype 2, 2 samples tested positive for serotype 5, 1 for serotype 1 and 1 for serotype 4 (See Supplementary material Table S2).

Discussion

We have developed AHSV TS RT-qPCR assays that were then characterized for applicability and practicability (Broeders et al., 2014) using a large group of blood samples collected from horses that were naturally infected with AHSV. All 9 serotypes 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.

Application 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 was not monotypic (serotype 3). The PI test used sera generated using the 1995 reference strains. Although cross reactivity occurs between serotypes (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 serotype 3 includes serotype 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 serotype 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 serotype 3 field samples. Interestingly, the live attenuated AHSV serotype 3 vaccine strain derived from this AHSV serotype 3 reference strain has recently been shown to be a reassortant between AHSV serotypes 1 and 3 (Guthrie et al., 2015) suggesting that the prototype virus stock from which this vaccine strain was derived contained viruses of both serotype 1 and 3.

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 serotypes are available and monotypic antisera have been generated such studies cannot be completed but further characterisation of these assays using appropriate methods (Broeders et al., 2014) is warranted.

Concurrently with our development of AHSV TS RT-qPCR assays, Bachanek-Banowska et al (2014) 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 serotypes, field

strains representing six of the nine AHSV serotypes, and 4 vaccine strains. Relevant to all AHSV TS RT-qPCR assays, Manole et al. (2012) 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 neutralization 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 (2014) 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 (Anon., 2012). 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 (Weyer et al., 2013; Quan et al., 2010). Importantly, virus serotype 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 (Grewar et al., 2013).

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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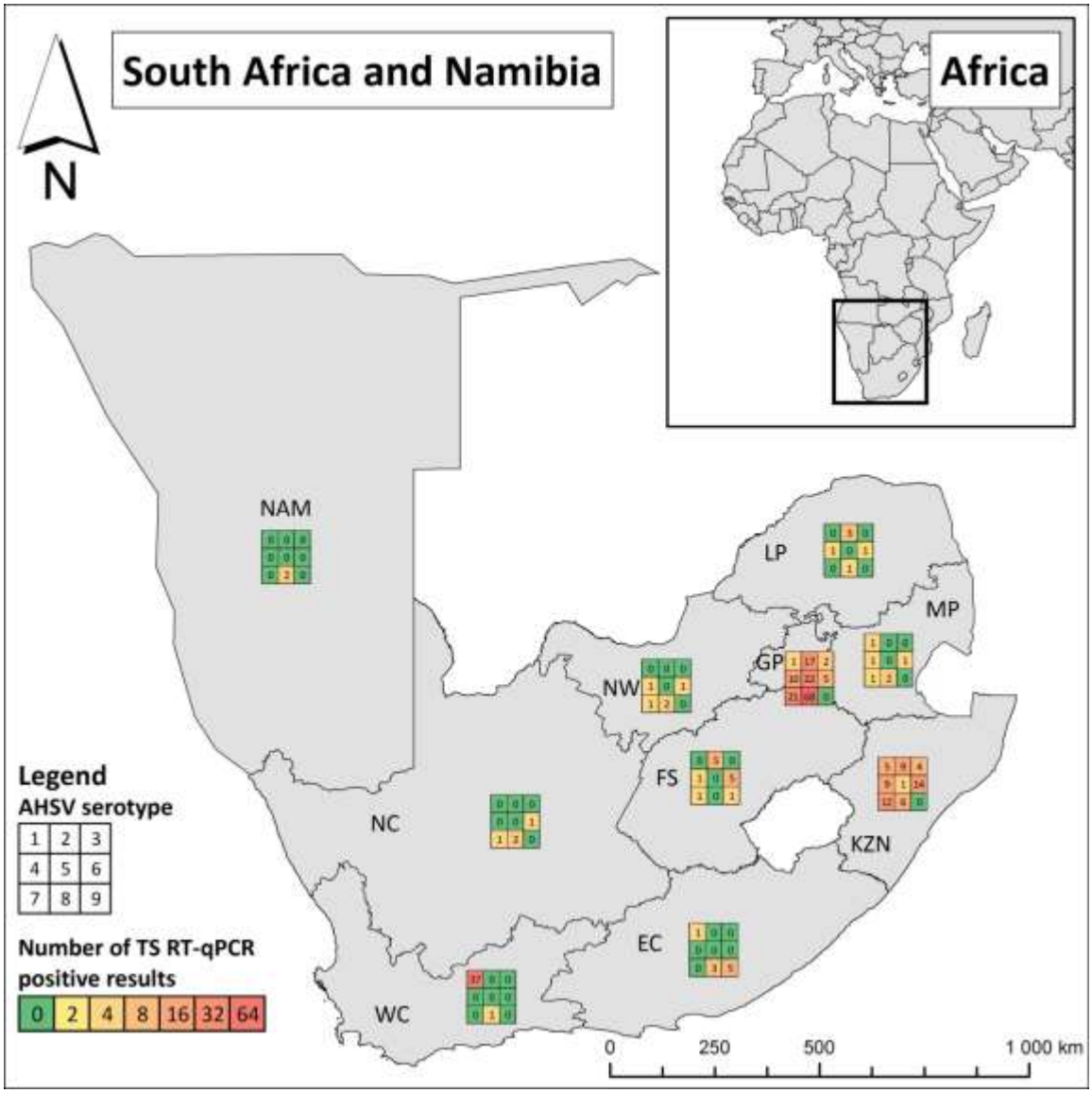
Supporting Information



Supplementary Figure S1: 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.

Supplementary Table S1: 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	*	*	*	*	*	*	*
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	*	*	*	*	*

Laboratory Reference	VI	PI	AHSV RT-qPCR Cq	AHSV TS RT-qPCR Cq								
				1	2	3	4	5	6	7	8	9
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	*
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	*

Laboratory Reference	VI	PI	AHSV RT-qPCR Cq	AHSV TS RT-qPCR Cq								
				1	2	3	4	5	6	7	8	9
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	*	*	*	*	*	*	*
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	*	*	*	*	*	*	*

Laboratory Reference	VI	PI	AHSV RT-qPCR Cq	AHSV TS RT-qPCR Cq								
				1	2	3	4	5	6	7	8	9
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	*	*
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	*	*

Laboratory Reference	VI	PI	AHSV RT-qPCR Cq	AHSV TS RT-qPCR Cq								
				1	2	3	4	5	6	7	8	9
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	*

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	*

Laboratory Reference	VI	PI	AHSV RT-qPCR Cq	AHSV TS RT-qPCR Cq								
				1	2	3	4	5	6	7	8	9
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

Supplementary Table S2: 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