Development and optimisation of a duplex real-time reverse transcription quantitative PCR assay targeting the VP7 and NS2 genes of African horse sickness virus

M. Quan[a,b], C.W. Lourens[a,b], N.J. MacLachlan[c], I.A. Gardner[d] and A.J. Guthrie[a]

[a] Equine Research Centre and [b] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, SOUTH AFRICA.
[c] Equine Viral Disease Laboratory, Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA.
[d] Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA.

Corresponding author: Melvyn Quan, Equine Research Centre, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, SOUTH AFRICA. Tel: +27-12-5298142, Fax: +27-12-5298312, E-mail: melvyn.quan@up.ac.za

Abstract

Nucleotide sequences of 52 South African isolates of African horse sickness virus (AHSV) collected during 2004 – 2005 and including viruses of all nine AHSV serotypes, were used to design and develop a duplex real-time reverse transcription quantitative PCR (RT-PCR) assay targeting the VP7 (S8) and NS2 (S9) genes of AHSV. The assay was optimized for detection of AHSV in fresh and frozen blood of naturally infected horses. Assay performance was enhanced using random hexamers rather than gene specific primers for RT, and with denaturation of double-stranded RNA in the presence of random hexamers. The assay was efficient with a linear range of at least five orders of magnitude. The analytical sensitivity of the assay was 132 copies of the target genes (4,125 copies per ml of blood), and the assay was at least ten-fold more sensitive than virus isolation on BHK-21 cells. The assay was also highly specific because it did not detect related orbiviruses, such as bluetongue and equine encephalosis viruses.

Keywords: African horse sickness virus, real-time quantitative RT-PCR, VP7 gene, NS2 gene, duplex

1. Introduction

African horse sickness (AHS) is caused by *African horse sickness virus* (AHSV), which belongs to the genus *Orbivirus*, family *Reoviridae*. AHS is a non-contagious disease of equids, transmitted by *Culicoides* midges (Diptera: Ceratopogonidae). In the equine host, the virus replicates in endothelial cells and macrophages, and causes vascular injury that can result in four clinical forms of AHS, i.e. pulmonary (dunkop), cardiac (dikkop), mixed and horse sickness fever forms (Guthrie and Quan, 2009).

AHS is an OIE-listed disease because of its associated high mortality and potential for rapid international spread (OIE, 2005). An outbreak of AHS in a naïve horse population can be devastating, with cumulative mortality of up to 95% (Guthrie and Quan, 2009; Mirchamsy and Hazrati, 1973). The disease is endemic to sub-Saharan Africa and outbreaks have been reported recently in Ethiopia, Senegal, Ghana, Nigeria and southern Africa to the World Organisation for Animal Health (OIE). AHS has spread previously from the African continent to Europe in 1966 and 1987 to 1990 (Díaz-Montilla and Paños Martí, 1967; Rodriguez et al., 1992), as well as to the Middle East/south west Asia on several occasions (Alexander, 1948; Anderson et al., 1989; Howell, 1960; Reid, 1961). There is concern currently regarding the potential for AHS to re-emerge from Africa because of the effects of climate change (Dufour et al., 2008; Gale et al., 2010). Climate change has been proposed to be responsible for the recent spread of bluetongue virus into Europe from Africa and Asia (Purse et al., 2005).

Accurate diagnostic assays i) demonstrate freedom from infection in a defined population, ii) certify freedom from infection or agent in individual animals or products, iii) assist in eradication of infection from a defined population or, iv) confirm diagnosis of suspect or clinical cases (OIE, 2008). For all purposes, accurate diagnosis of AHSV is essential due to the severe nature of the disease and the implications of a false negative result. A rapid assay is desirable, and compared to traditional viral isolation techniques, molecular methods based on the reverse transcription polymerase chain reaction (RT-PCR) have dramatically shortened the time required to identify pathogens.

The majority of previously published AHSV assays are based on visualisation of the PCR product on an agarose gel. They have been designed to target either the VP3

(L3) (Aradaib, 2009; Aradaib et al., 2006; Sakamoto et al., 1994), VP7 (S8) (Moulay et al., 1995; Sailleau et al., 1997; Zientara et al., 1993; Zientara et al., 1994; Zientara et al., 1995b; Zientara et al., 1998), NS1 (M5) (Mizukoshi et al., 1994), NS2 (S9) (Bremer and Viljoen, 1998; Stone-Marschat et al., 1994) or NS3 (S10) (Zientara et al., 1995a; Zientara et al., 1995b) gene. With real-time RT-PCR, the PCR product is detected in "real time". The advantages over gel-based assays are an increased analytical sensitivity and a more rapid assay with less potential for contamination. Real-time RT-PCR assays for AHSV have been published recently and target either the VP7 (Agüero et al., 2008; Fernández-Pinero et al., 2009) or NS1 (Rodriguez-Sanchez et al., 2008) gene.

This paper describes the development and optimisation of a duplex real-time reverse transcription quantitative PCR (RT-qPCR) assay for the sensitive and specific detection of AHSV in blood samples from horses naturally infected with the virus. None of the previously published AHSV assays have been optimised for the detection of AHSV in blood samples from naturally infected horses. Detection of AHSV in blood, rather than from tissue culture, is more challenging, due to the presence of PCR inhibitors (e.g. haemoglobin) and the low concentration of AHSV in the blood of some infected horses. The assay is novel in that it targets both the VP7 and NS2 genes of AHSV and is quantitative. Critical control parameters of the assay, as well as the repeatability, analytical sensitivity and specificity of the assay were estimated.

2. Material and Methods

2.1. Sequencing

AHSV was isolated from 52 samples submitted in 2004 and 2005 to the Equine Virology Research Laboratory, Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science, University of Pretoria, by culture on BHK-21 cells and typed by the plaque inhibition neutralization test (Quan et al., 2008). Nine reference strains (designated as "Jane" 1-9), derived from virulent field viruses and representing each AHSV serotype (one to nine), were included in the isolates selected for sequencing. Terminal primers for the VP7 (Zientara et al., 1993), NS1 and NS2 genes were designed with the aid of FastPCR 5.2.12 (Table 1a) and sequences available on the National Center for Biotechnology Information's GenBank website (www.ncbi.nlm.nih.gov). RT-PCR and direct sequencing of the

PCR product were performed as described previously (Quan et al., 2008). Phred (Ewing et al., 1998; Ewing and Green, 1998) and the Staden package (Staden, 1996; Staden et al., 2000) were used for base calling and sequence assembly. Sequences were aligned using MAFFT (Katoh et al., 2002; Katoh et al., 2005), conserved regions identified with the use of quality scores in ClustalX (Figure 1) and PrimerExpress 2.0 (Applied Biosystems, Foster City, USA) software used to design primers and TaqMan® MGB™ hydrolysis probes. Pairwise genetic distances were calculated from nucleotide substitution models determined using Modeltest v3.7 (Posada and Crandall, 1998) and PAUP* v4b10 (Swofford, 2003).

2.2. Optimisation

All steps of the assay, i.e. processing of the blood samples, RNA extraction, RT and PCR were optimised (details supplied in supplementary information). Optimisation was first carried out on the LightCycler® 2.0 Real-Time PCR System (Roche, Mannheim, Germany) and then on the Step-One™ Plus Real-Time PCR System (Applied Biosystems). The criterium for determining the protocol was based on the method that resulted in the lowest cycle threshold (C_T) values or the highest efficiency of the PCR.

Individual steps of the method that were optimised were: i) use of fresh or frozen blood samples, ii) testing of three different RNA extraction kits (RNeasy Mini Kit, QIAamp RNA Blood Mini Kit and QIAamp Viral Mini Kit, all Qiagen, Hilden, Germany), iii) denaturation method, iv) temperature of the reverse transcription (RT), v) testing of three different RT kits [Reverse Transcription Reagents (Applied Biosystems), Transcriptor First Strand cDNA Synthesis kit (Roche) and MessageSensor™ RT Kit (Ambion, Austin USA)], vi) length of the RT, vii) use of random hexamers or gene specific primers for RT, viii) testing of three different PCR kits (TaqMan® Gene Expression Master Mix, TaqMan® Universal PCR Master Mix and Fast Universal PCR Master Mix, all Applied Biosystems), ix) optimisation of primer and probe concentration, and x) duplex conditions.

2.3. AHSV standard dsRNA

The VP7 and NS2 PCR products from the real-time RT-PCR assay were purified using a QIAquick® PCR Purification Kit (Qiagen), cloned using the pGEM®-T Easy Vector System II (Promega, Madison, USA), according to the manufacturer's

instructions and sequenced using T7 and SP6 primers to determine the orientation of the insert and to check for sequence differences (Quan et al., 2008).

Aliquots of plasmid DNA were linearized separately with *Sal*1 and *Nco*1 restriction enzymes (Promega). The MEGAshortscript^{TM} kit (Ambion) was used for transcription of *Sal*1 linearized plasmid and the MEGAscript^{TM} SP6 kit (Ambion) used for *Nco*1 linearized plasmid according to the manufacturer's instructions.

RNA quantity was determined using both a NanoDrop^{TM} (Thermo Fisher Scientific, Waltham, USA) and a DU$^{®}$530 (Beckman Coulter, Brea, USA) UV spectrophotometer on an extinction coefficient setting of 33. Results from both spectrophotometers were averaged, the molecular weight of the transcripts calculated (www.ambion.com/techlib/misc.oligo_calculator.html) and the copy number of transcripts per µl calculated. Equal concentrations of T7 and SP6 generated RNA were combined, heated to 95°C for two min and cooled to room temperature to form dsRNA. Aliquots of a ten-fold dilution series of 10^8 to 10^0 dsRNA copies/µl were prepared using TE buffer (Ambion) as a diluent and stored at -80°C until needed.

2.4. Within- and between-day repeatability of the assays

Each dilution of the VP7 and NS2 dsRNA dilution series was combined in the same well and analysed using the same protocol used for blood samples. No-RT controls were included to assess the efficiency of the DNase step in preparing the dsRNA. The dilution series was run in triplicate on six separate days (total 18 runs). Within-day and between-day variation of the assay was assessed with analysis of variance (ANOVA) and coefficient of variation (CV).

2.5. Efficiencies of the assay, analytical sensitivity and specificity

The efficiency of each assay was calculated from the slope of the linear regression of the C_T data against log dilution of the dilution series using the formula: efficiency = $[10^{(-1/slope)}] - 1$. Analytical sensitivity (defined as the viral copy number detected 95% of the time) of each assay was calculated from a sigmoidal regression of sensitivity against log dilution (Bhoora et al., 2009; Sibeko et al., 2008). The sensitivity of each assay was compared to the sensitivity of AHSV isolation on BHK-21 cells (Quan et al., 2008). Three AHSV positive tissue culture samples (serotypes six, five and two), at a concentration of $10^{4.8}$ $TCID_{50}$/ml, were diluted ten-fold in a dilution series up to a

10^{-9} dilution, and 0.5 ml of each dilution tested in parallel by viral isolation on BHK-21 cells (Quan et al., 2008) and RT-qPCR. Analytical specificity of each assay was determined by testing the assay on tissue cultures of various orbiviruses (bluetongue virus, equine encephalosis virus, epizootic hemorrhagic disease virus and Palyam virus). RNA was extracted from 100 µl of tissue culture suspension of the orbivirus using the RNeasy Mini kit (Qiagen) and eluted in 50 µl water, before running the assays.

2.6. Protocol

Whole blood (500 µl) in EDTA/heparin was transferred to an eppendorf tube, frozen and thawed. The sample was vortexed briefly and spun at 15,000 *g* in a benchtop centrifuge for ten min, which resulted in the separation of the sample into three layers: a clear top layer, opaque middle layer and a pellet at the bottom of the tube. Both top and middle layers were removed and 900 µl H_2O added to the pellet. The sample was vortexed briefly, spun at 7,000 *g* for five min, the supernatant removed, 350 µl of RLT buffer (RNeasy Mini Kit, Qiagen) added and the sample vortexed until the pellet was dissolved. A volume of 350 µl of 70% ethanol was added and the rest of the extraction carried out as described in the Qiagen RNeasy Mini Kit protocol. The sample was eluted in 30 µl RNase free H_2O.

RNA (7.7 µl) was transferred to a well in a 96-well plate or a PCR tube containing 1 µl of random hexamers (50 µM, Applied Biosystems) and the plate/tube centrifuged briefly to collect the contents in the bottom of the well/tube. The sample was heated to 95°C for one min in a PCR machine and transferred immediately onto ice. Reverse Transcription Reagents (Applied Biosystems) were added to each sample at the following final concentrations: 5.5 mM $MgCl_2$, 0.5 mM of each dNTP, 8 U RNase inhibitor and 25 U Multiscribe™ Reverse Transcriptase in a 20 µl total volume. The sample was centrifuged briefly and heated to 42°C for 30 min, 95°C for five min and held at 4°C in a PCR machine.

For analysis on a Step One Plus PCR machine (Applied Biosystems), 5 µl of cDNA was added to TaqMan® Fast Universal PCR Master Mix reagents (Applied Biosystems), to obtain final primer and probe concentrations of 300 nM/primer and 250 nM/probe in a 20 µl total volume. The sample was centrifuged briefly and run on the PCR machine using the default fast programme (40 cycles of 95°C for one sec, 60°C for 20 sec).

For analysis on a LightCycler® PCR machine (Roche), 5 µl of cDNA was added to LightCycler® TaqMan® Master reagents (Roche), to obtain final primer and probe concentrations of 500 nM/primer and 100 nM/probe in 20 µl total volume. The following cycling programme was used: 95°C for ten min, 40 cycles of 95°C for ten sec, 60°C for 30 sec and 72°C for one sec. After cycling, the sample was cooled at 40°C for 30 sec.

2.7. Serial sampling of an AHSV infected horse

A Boerperd horse was inoculated intravenously with $10^{5.5}$ $TCID_{50}$ of a virulent field strain of AHSV, serotype four. Serial blood samples were collected daily for 28 days and then three times a week for 100 days post inoculation. Samples were collected in EDTA Vacutainer® tubes and analyzed by RT-qPCR. The procedure was approved by the University of Pretoria Animal Use and Care Committee as part of a separate study (Guthrie et al., 2009)).

2.8. Data analysis

Data were analysed with either SPSS 15.0 or 17.0 for Windows (SPSS Inc., Chicago, USA).

3. Results

3.1. Sequencing

The AHSV isolates that were sequenced were obtained from South Africa, Namibia and Zimbabwe. At least three AHSV isolates per serotype were sequenced and 43, 42 and 37 unique VP7, NS1 and NS2 gene sequences, respectively, were obtained (Genbank accession nos **xxxx**) (Table 2). Although the VP7, NS1 and NS2 genes were well conserved, no completely (100%) conserved region in the NS1 gene was identified. In contrast, conserved regions within the VP7 and NS2 gene were identified (Figure 1) and assays targeting only the VP7 and NS2 genes and using TaqMan® MGB™ hydrolysis probes were designed (Table1b).

The VP7, NS1 and NS2 gene sequences (together with Genbank sequences) were used to align primer and probe sequences from published AHSV assays and all the

assays showed mismatches in either, or both, the primer and probe regions (Figure 2).

3.2. Optimisation

Details of the optimisation are provided in the supplementary information, but in brief, i) no significant difference was detected between processing fresh or frozen blood samples, ii) extraction of RNA using the RNeasy Mini Kit provided the most consistent results, iii) there was a 1.3-fold increase in amount of AHSV detected when heat (95°C), instead of methylmercury(II) hydroxide (Alfa Aesar, London, UK) was used to denature the dsRNA and significantly lower C_T value's were obtained when dsRNA was denatured at 95°C for one min compared to 98°C for five min, iv) the addition of random hexamers to dsRNA before denaturation resulted in an 18-fold higher detection of AHSV (a mean decrease in C_T of 4.16) compared to the addition of random hexamers after denaturation, v) the analytical sensitivity of the assays increased 100-fold when random hexamers, instead of gene-specific primers were used for RT, vi) the use of Reverse Transcription Reagents at 42°C for 30 min was determined to be the optimum kit, temperature and time for RT, vi) the TaqMan® Fast Universal Kit, using a final primer and probe concentration of 300 nM and 250 nM, respectively, was the optimum kit and concentration of primers and probe for PCR, and vii) significantly lower C_T's (mean difference of 0.33 for the VP7 assay and 0.22 for the NS2 assay) were obtained when the assay was run as a singleplex compared to a duplex assay.

3.3. Standard curve

The DNA concentration in the RNA standard was approximately $10^{4.6}$ (38,000 or 15 C_T difference) and $10^{4.3}$ (22, 000 or 14 C_T difference) times less than the RNA concentration for the VP7 and NS2 standards, respectively. DNA contamination of the RNA standard was therefore regarded as not of practical consequence.

Linear regressions of serial dilutions of AHSV RNA are shown in Figure 3. The linear dynamic range covers six $\log_{10}$ concentrations (10^2 – 10^8 RNA copies) for the VP7 assay and five $\log_{10}$ concentrations (10^2 – 10^7 RNA copies) for the NS2 assay.

3.4. Within- and between-day repeatability of the assays

ANOVA showed no significant daily variation in the slope ($p = 0.627$) or intercept ($p = 0.437$) for the VP7 assay, but there was a significant between-run variation in slope ($p = 0.010$) and intercept ($p = 0.044$) for the NS2 assay, due to the high CV of the 10^8 AHSV RNA copies data (Figure 4b). A repeat of the analysis excluding these data was not significant and further calculations were carried out with these data excluded. Within-run, the lowest CV's were obtained for the high AHSV concentration samples, increasing with decreasing AHSV concentrations.

3.5. Efficiencies of the assays, analytical sensitivity and specificity

The PCR efficiencies of the VP7 and NS2 assays were 111% ($\sigma = 0.05$, $n = 18$) and 99% ($\sigma = 0.06$, $n = 18$) respectively. The analytical sensitivities of the assays were 132 (VP7) and 114 (NS2 assay) AHSV copies, or a C_T-value of 36.13 and 37.71, respectively (Figure 5). Calculated from the standard curve, this equates to $10^{3.62}$ (4,125) and $10^{3.55}$ (3,562) AHSV/ml blood for the VP7 and NS2 assays, respectively. The VP7 and NS2 assays were both ten times ($n = 3$) more sensitive than viral isolation and an estimated $10^{2.99}$ (987) AHSV particles were required to infect 50% of BHK-21 tissue culture plates (one $TCID_{50}$). The assays were specific, as fluorescence above the threshold (0.1) was detected for all nine serotypes of AHSV within 40 PCR cycles, but not for other orbiviruses (Table 3).

3.6. Serial sampling of an AHSV infected horse

AHSV viraemia was first detected seven days post-inoculation. The amount of AHSV in the blood increased rapidly and a peak viraemia of $10^{6.89}$ AHSV RNA/ml blood was detected 15 days post inoculation (Figure 6). AHSV dsRNA was present in the blood for up to 97 days post-inoculation, at a concentration that could be detected 95% of the time for up to 51 days post-inoculation.

4. Discussion

The real-time RT-PCR assay described is unique in that it is designed from the sequences of current circulating field strains of AHSV. Sequencing of southern African AHSV isolates showed that the NS2 gene was the most conserved gene of the three genes that were evaluated (VP7, NS1, NS2), as judged by the median pairwise genetic distance between sequences, followed in order by the NS1 and VP7 genes. All three genes were highly conserved compared to the NS3 gene, the

second most variable gene in the AHSV genome (Van Niekerk et al., 2003), as the maximum variation between amino acid sequences was less than 3% for the VP7, NS1 and NS2 proteins, whereas there was as much as 36% variation between amino acid sequences of the various NS3 proteins (Table 2).

Evaluation of sequence data obtained from southern African AHSV isolates and GenBank with published real-time RT-PCR assays showed nucleotide mismatches in the primer/probe regions, with differences in either the forward primer (Agüero et al., 2008), probe (Fernández-Pinero et al., 2009), or both primers and probe (Rodriguez-Sanchez et al., 2008). The ability of these assays to detect all southern African AHSV strains is not known, but it is possible that sensitivity for some of the assays may be reduced due to mismatches in either the centre (Fernández-Pinero et al., 2009) or at both the 5' and 3' ends (Rodriguez-Sanchez et al., 2008) of the probe region.

A protocol for the detection of AHSV in whole blood was optimised. Freezing of blood samples was convenient, as samples could be stored and batched before the assay was run and the freezing process did not result in a significant loss of template. To extract RNA from the thawed samples, the QIAamp Viral kit was the most sensitive of the extraction kits tested, however, the results were not consistent, presumably as a result of inefficient removal of PCR inhibitors such as haemoglobin from the eluate. The QIAamp Viral kit was designed for extraction of RNA from cell-free material (serum/plasma), whereas our assay required the extraction of RNA from a cell pellet with a higher concentration of PCR inhibitors than serum/plasma. An added advantage of the RNeasy Mini kit was that the kit could also be used for extracting AHSV RNA from organ samples collected at post mortems. Thus, the RNeasy Mini kit was selected because of more consistent results that were obtained using it. It took approximately five hours to get results from 15 samples and the kit was therefore suitable for low-throughput situations, such as confirmation of an AHS diagnosis. For high-throughput situations, such as surveillance during an AHS outbreak where a large number of samples would need to be tested, automated extraction methods would need to be developed.

The step that that had the most significant effect on the sensitivity of the assay was the denaturation of dsRNA together with random hexamers. An 18-fold greater amount of AHSV was detected when dsRNA was denatured together with random hexamers. We attributed this difference to the hexamers' ability to bind to single-stranded heat-denatured RNA before the RNA had cooled sufficiently to reform

dsRNA. Another significant step was the use of random hexamers instead of gene-specific primers for RT. Although there was not a substantial difference in C_T results obtained when using either primer, the limit of detection was 100-fold greater when random hexamers were used, indicating the sensitivity of random hexamers in the RT of low concentrations of RNA. The assay is at least ten-fold more sensitive than virus isolation and specific, detecting only AHSV and no other orbiviruses.

Assessment of the repeatability of the assay showed high between-run CV's results from the high AHSV concentration samples, which may be due to inhibition of the RT or PCR by the high nucleic acid concentration or other inhibitors in these samples.

The assay is in a duplex format and therefore redundant. The redundancy insures against false-negative results due to nucleotide variation within any one of the target regions. A high degree of sequence variation of the AHSV genome can be expected because of the high mutation rate of RNA viruses (Belshaw et al., 2008; Drake et al., 1998). The assay is complementary to new recombinant vaccines expressing outer capsid proteins of AHSV (VP2 and VP5) (Guthrie et al., 2009). As the assay is specific for AHSV VP7 and NS2 genes, it can differentiate infected from vaccinated animals, something that is not possible with the current use of modified live vaccines.

In summary, a rapid, analytically sensitive and specific assay for the detection of AHSV is described. In addition to providing an important new diagnostic test, the assay is quantitative and the results suitable for mathematical modelling and pathogenesis studies, which will allow for a greater understanding of AHS. Validation of the diagnostic performance of the assay with field samples is the next step to ensure its utility.

5. Acknowledgments

This research was funded by the Equine Research Centre, University of Pretoria.

References

Agüero, M., Gómez-Tejedor, C., Angeles, C.M., Rubio, C., Romero, E., Jiménez-Clavero, A., 2008. Real-time fluorogenic reverse transcription polymerase chain reaction assay for detection of African horse sickness virus. J. Vet. Diagn. Invest. 20, 325-328.

Alexander, R.A., 1948. The 1944 epizootic of horsesickness in the Middle East. Onderstepoort J. Vet. Sci. Anim. Ind. 23, 77-92.

Anderson, E.C., Mellor, P., Hamblin, C., 1989. African horse sickness in Saudi Arabia. Vet. Rec. 125, 489.

Aradaib, I.E., 2009. PCR detection of African horse sickness virus serogroup based on genome segment three sequence analysis. J. Virol. Methods 159, 1-5.

Aradaib, I.E., Mohemmed, M.E., Sarr, J.A., Idris, S.H., Ali, N.O., Majid, A.A., Karrar, A.E., 2006. Short communication: a simple and rapid method for detection of African horse sickness virus serogroup in cell cultures using RT-PCR. Vet. Res. Commun. 30, 319-324.

Belshaw, R., Gardner, A., Rambaut, A., Pybus, O.G., 2008. Pacing a small cage: mutation and RNA viruses. Trends Ecol. Evol. 23, 188-193.

Bhoora, R., Quan, M., Franssen, L., Butler, C.M., Van der Kolk, J.H., Guthrie, A.J., Zweygarth, E., Jongejan, F., Collins, N.E., 2010. Development and evaluation of real-time PCR assays for the quantitative detection of *Babesia caballi* and *Theileria equi* infections in horses from South Africa. Vet. Parasitol. doi:10.1016/j.vetpar.2009.11.011

Bremer, C.W., Viljoen, G.J., 1998. Detection of African horsesickness virus and discrimination between two equine orbivirus serogroups by reverse transcription polymerase chain reaction. Onderstepoort J. Vet. Res. 65, 1-8.

Díaz-Montilla, R., Paños Martí, P., 1967. Epizootiologia de la peste equina en españa. Bull. OIE 68, 705-714.

Drake, J.W., Charlesworth, B., Charlesworth, D., Crow, J.F., 1998. Rates of spontaneous mutation. Genetics. 148, 1667-1686.

Dufour, B., Moutou, F., Hattenberger, A.M., Rodhain, F., 2008. Global change: impact, management, risk approach and health measures - the case of Europe. Rev. Sci. Tech. 27, 529-550.

Ewing, B., Green, P., 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. 8, 186-194.

Ewing, B., Hillier, L., Wendl, M.C., Green, P., 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. 8, 175-185.

Fernández-Pinero, J., Fernández-Pacheco, P., Rodríguez, B., Sotelo, E., Robles, A., Arias, M., Sánchez-Vizcaíno, J.M., 2009. Rapid and sensitive detection of African horse sickness virus by real-time PCR. Res. Vet. Sci. 86(2), 353-358.

Gale, P., Brouwer, A., Ramnial, V., Kelly, L., Kosmider, R., Fooks, A.R., Snary, E.L., 2010. Assessing the impact of climate change on vector-borne viruses in the EU through the elicitation of expert opinion. Epidemiol. Infect. 138, 214-225.

Guthrie, A.J., Quan, M., 2009. African horse sickness, in: Mair, T.S., Hutchinson, R.E. (Eds.), Infectious diseases of the horse, 1st edn. Equine Veterinary Journal Ltd., Ely, pp. 72-82.

Guthrie, A.J., Quan, M., Lourens, C.W., Audonnet, J.C., Minke, J.M., Yao, J., He, L., Nordgren, R., Gardner, I.A., MacLachlan, N.J., 2009. Protective immunization of horses with a recombinant canarypox virus vectored vaccine co-expressing genes encoding the outer capsid proteins of African horse sickness virus. Vaccine 27, 4434-4438.

Howell, P.G., 1960. The 1960 epizootic of African horsesickness in the Middle East and S.W. Asia. J. S. Afr. Vet. Med. Assoc. 31, 329-334.

Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. 33, 511-518.

Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059-3066.

Mirchamsy, H., Hazrati, A., 1973. A review on aetiology and pathogeny of African horsesickness. Arch. Inst. Razi. 25, 23-46.

Mizukoshi, N., Sakamoto, K., Iwata, A., Ueda, S., Kamada, M., Fukusho, A., 1994. Detection of African horsesickness virus by reverse transcriptase polymerase chain reaction (RT-PCR) using primers for segment 5 (NS1 gene). J. Vet. Med. Sci. 56, 347-352.

Moulay, S., Zientara, S., Sailleau, C., Cruciere, C., 1995. Detection of African horse sickness viruses by dot-blot hybridization using a digoxigenin-labelled probe. Mol. Cell Probes 9, 233-237.

OIE, 2005. Appendix XXVIII: Report of the meeting of the OIE ad hoc group on diseases/pathogenic agent notification on a new OIE list of terrestrial animal disease. In: Report of OIE Terrestrial Animal Health Standards Commission/January 2005. pp. 495-515: OIE.

OIE, 2008. OIE quality standard and guidlines for veterinary laboratories: infectious diseases. OIE, Geneva.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics. 14, 817-818.

Purse, B.V., Mellor, P.S., Rogers, D.J., Samuel, A.R., Mertens, P.P., Baylis, M., 2005. Climate change and the recent emergence of bluetongue in Europe. Nat. Rev. Microbiol. 3, 171-181.

Quan, M., van Vuuren, M., Howell, P.G., Groenewald, D., Guthrie, A.J., 2008. Molecular epidemiology of the African horse sickness virus S10 gene. J. Gen. Virol. 89, 1159-1168.

Reid, N.R., 1961. African horse sickness. Br. Vet. J. 118, 137-142.

Rodriguez, F., Oliver, J.L., Marin, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitution. J. Theor. Biol. 142, 485-501.

Rodriguez, M., Hooghuis, H., Castaño, M., 1992. African horse sickness in Spain. Vet. Microbiol. 33, 129-142.

Rodriguez-Sanchez, B., Fernandez-Pinero, J., Sailleau, C., Zientara, S., Belak, S., Arias, M., Sanchez-Vizcaino, J.M., 2008. Novel gel-based and real-time PCR assays for the improved detection of African horse sickness virus. J. Virol. Methods 151, 87-94.

Sailleau, C., Moulay, S., Cruciere, C., Laegreid, W.W., Zientara, S., 1997. Detection of African horse sickness virus in the blood of experimentally infected horses: comparison of virus isolation and a PCR assay. Res. Vet. Sci. 62, 229-232.

Sakamoto, K., Punyahotra, R., Mizukoshi, N., Ueda, S., Imagawa, H., Sugiura, T., Kamada, M., Fukusho, A., 1994. Rapid detection of African horsesickness virus by the reverse transcriptase polymerase chain reaction (RT-PCR) using the amplimer for segment 3 (VP3 gene). Arch. Virol. 136, 87-97.

Sibeko, K.P., Oosthuizen, M.C., Collins, N.E., Geysen, D., Rambritch, N.E., Latif, A.A., Groeneveld, H.T., Potgieter, F.T., Coetzer, J.A., 2008. Development and evaluation of a real-time polymerase chain reaction test for the detection of *Theileria parva* infections in Cape buffalo (*Syncerus caffer*) and cattle. Vet. Parasitol. 155, 37-48.

Staden, R., 1996. The Staden sequence analysis package. Mol. Biotechnol. 5, 233-241.

Staden, R., Beal, K.F., Bonfield, J.K., 2000. The Staden package, 1998. Methods Mol. Biol. 132, 115-130.

Stone-Marschat, M., Carville, A., Skowronek, A., Laegreid, W.W., 1994. Detection of African horse sickness virus by reverse transcription-PCR. J. Clin. Microbiol. 32, 679-700.

Swofford, D.L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4b10. Sinauer Associates, Sunderland, Massachusetts.

Van Niekerk, M., Freeman, M., Paweska, J.T., Howell, P.G., Guthrie, A.J., Potgieter, A.C., van Staden, V., Huismans, H., 2003. Variation in the NS3 gene and protein in South African isolates of bluetongue and equine encephalosis viruses. J. Gen. Virol. 84, 581-590.

Zientara, S., Sailleau, C., Moulay, S., Cruciere, C., 1994. Diagnosis of the African horse sickness virus serotype 4 by a one-tube, one manipulation RT-PCR reaction from infected organs. J. Virol. Methods 46, 179-188.

Zientara, S., Sailleau, C., Moulay, S., Cruciere, C., 1995a. Differentiation of African horse sickness viruses by polymerase chain reaction and segments 10 restriction patterns. Vet. Microbiol. 47, 365-375.

Zientara, S., Sailleau, C., Moulay, S., Cruciere, C., el-Harrak, M., Laegreid, W.W., Hamblin, C., 1998. Use of reverse transcriptase-polymerase chain reaction (RT-PCR) and dot-blot hybridisation for the detection and identification of African horse sickness virus nucleic acids. Arch. Virol. Suppl. 14, 317-327.

Zientara, S., Sailleau, C., Moulay, S., Plateau, E., Cruciere, C., 1993. Diagnosis and molecular epidemiology of the African horsesickness virus by the polymerase chain reaction and restriction patterns. Ann. Rech. Vet. 24, 385-395.

Zientara, S., Sailleau, C., Moulay, S., Wade-Evans, A., Cruciere, C., 1995b. Application of the polymerase chain reaction to the detection of African horse sickness viruses. J. Virol. Methods 53, 47-54.

Figure legends

Figure 1. Plot of conservation of sites on the **(a)** VP7, **(b)** NS2 and **(c)** NS1 genes. No column at a nucleotide position indicates 100% conservation, the higher the column, the greater the nucleotide variation at that site. Conserved regions are indicated by grey blocks; no conserved region is evident in the NS1 gene.

Figure 2. AHSV RT-PCR assay primers (unshaded arrows) and/or probe (shaded arrow) sequences of published assays **(a – d, f, g, i, j)** aligned with 61 VP7 **(a – e)**, 58 NS2 **(f – h)** and 68 NS1 **(i.j)** gene sequences. Dots indicate identity with the first sequence in the group. Identical sequences are represented only once in the figure and labelled with either the accession number (normal text) or name (italics) of a randomly selected sequence in the group, followed by the number of sequences in brackets. * Sequence used for the numbering of the nucleotides.

Figure 3. Linear regression analysis of a serial dilution of AHSV **a)** VP7 and **b)** NS2 RNA copies (n = 18). Triangular data points not included in the regression analysis.

Figure 4. Analytical sensitivities of the AHSV **a)** VP7 and **b)** NS2 assays (± 95% confidence intervals) with fitted sigmoidal regressions equations.

Figure 5. Coefficient of variation (CV) of AHSV **a)** VP7 and **b)** NS2 assays, calculated by running an AHSV standard curve daily in triplicate (within run) over six separate days (between run).

Figure 6. Development of viraemia in a Boerperd horse inoculated intravenously with $10^{5.5}$ $TCID_{50}$ of a virulent field strain of AHSV serotype 4, as determined by RT-qPCR targeting the VP7 and NS2 genes of AHSV. Negative RT-qPCR results were assigned a value of 10^2 AHSV/ml blood. The horizontal line indicates the virus concentration above which samples would test positive 95% of the time if AHSV were present in the blood.

SUPPLEMENTARY INFORMATION

Materials and methods

Processing blood samples

Naturally AHSV-infected whole blood (500 µl) was spun at 1,000 *g* in a benchtop centrifuge for ten min. The plasma was removed and the pellet resuspended in 900 µl phosphate-buffered saline. The eppendorf was inverted a few times to mix the contents and then spun at 1,000 *g* for ten min. The supernatant was removed, 500 µl H_2O added and the contents vortexed. The sample was spun at 15,000 *g* for ten min and the supernatant removed. The pellet was washed in 900 µl H_2O, spun at 7,000 *g* for five min and the supernatant discarded.

This method was compared to freezing blood: whole blood (500 µl) was frozen and thawed, spun at 15,000 *g* for ten min and the supernatant removed. The pellet was washed in 900 µl H_2O, spun at 7,000 *g* for five min and the supernatant discarded.

RNA extraction kits

The efficiency of three RNA extraction kits: RNeasy Mini Kit, QIAamp Viral RNA Mini Kit and QIAamp RNA Blood Mini Kit (all Qiagen, Hilden, Germany) to isolate RNA from washed and pelleted red blood cell membranes, obtained from naturally AHSV-infected horses, was compared. The RNA concentration in the eluate was measured with a NanoDrop™ 1000 Spectrophotometer.

dsRNA denaturation, temperature of reverse transcription

The efficiency of dsRNA denaturation was assessed by extracting dsRNA from two tissue culture (BHK-21 cells) viral isolates with the RNeasy Mini Kit and denaturing a ten-fold serial dilution series (10^{-1} – 10^{-8}) of dsRNA with either methylmercury(II) hydroxide (MMOH) (Alfa Aesar, London, UK) or heat.

To denature dsRNA with MMOH, one µl 0.2 M MMOH was added to five µl of dsRNA and incubated for ten min at room temperature. The mixture was reduced with one µl 1 M 2-mercaptoethanol (Sigma, St Louis, USA). To denature dsRNA with heat, five µl

RNA was added to two µl H_2O, heated to 98°C for five min and put immediately on ice.

To evaluate the influence of secondary RNA structure on the efficiency of reverse transcription, the denaturation method was followed by running the reverse transcription at various temperatures. The effect of secondary RNA structure on transcription would be more evident at lower temperatures. TaqMan® Reverse Transcription Reagents master mix (Applied Biosystems, Foster City, USA) was added to the denatured RNA to a final volume of 20 µl, incubated for 25°C for ten min, and then heated to either 42, 47 or 52°C for 30 min, before the temperature was raised to 95°C for five min.

Linear regression equations for each dilution series were obtained. A univariate analysis of variance (ANOVA) was used to perform a two factor ANOVA - slope and intercept of the linear regressions were used as the dependent variables and denaturation method and temperature of the reverse transcription (RT) as fixed factors.

Addition of one µl 50 µM random hexamers (Applied Biosystems) to dsRNA before denaturation was compared to denaturation of dsRNA alone (in this case the random hexamers were included in the RT-master mix). Subsequent to this experiment, denaturation of dsRNA (together with random hexamers) by heating to 98°C for five min was compared to denaturation at 95°C for one min.

Reverse transcription

Different RT kits were evaluated by extracting dsRNA from three tissue culture (BHK-21 cells) viral isolates with the RNeasy Mini Kit. A ten-fold serial dilution series (10^{-0} – 10^{-7}) of dsRNA was reverse transcribed with Reverse Transcription Reagents (Applied Biosystems), the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) and the MessageSensor™ RT Kit (Ambion, Austin, USA). RT was performed according to the manufacturer's instructions using random hexamers. PCR was performed using the VP7 assay and linear regression equations for each dilution series were obtained.

Using Reverse Transcription Reagents (Applied Biosystems), RT of a ten-fold serial dilution series (10^{-0} – 10^{-7}) of dsRNA was performed using both random hexamers

and gene specific primers (final concentration of 200 nM, primers were the same as those used in the real-time PCR assay) and the results compared.

Using Reverse Transcription Reagents (Applied Biosystems) and random hexamers, RT was performed for 30 and 60 min on positive field samples of AHSV and results compared.

PCR kits

Fourteen naturally AHSV-infected blood samples were reverse transcribed with both Applied Biosystems Reverse Transcription Reagents (42°C for 30 min and 95°C for 5 min) and High Capacity RNA-to-cDNA Master Mix (37°C for 60 min, 95°C for 5 min). The cDNA was used in a two-step RT-PCR assay to evaluate different Applied Biosystems PCR kits: TaqMan® Gene Expression Master Mix, TaqMan® TaqMan® Universal PCR Master Mix and Fast Universal PCR Master Mix (used according to the manufacturer's instructions). A further comparison between the latter two kits was then performed using 15 naturally AHSV-infected blood samples.

Gene specific primer concentrations were optimised by testing eight naturally AHSV-infected blood samples with a final primer concentration of 900, 300 and 50nM in the PCR reactions.

Gene specific probe concentrations were optimised by testing eight naturally AHSV-infected blood samples with a final probe concentration of 250, 150 and 50nM in the PCR reactions.

Duplexing of the AHSV assay was evaluated by testing 15 naturally AHSV-infected blood samples with VP7 and NS2 assays, and comparing the results to those obtained from a duplex assay containing the VP7 and the NS2 primers and probes in the same reaction.

Results

Processing blood samples

A paired t-test detected no significant differences [$p = 0.504$ (VP7 assay) and 0.257 (NS2 assay)] in C_T between processing fresh [$\bar{x} = 30.05$, $n = 11$ (VP7 assay); $\bar{x}$ =

28.40, n = 10 (NS2 assay)] and frozen [$\bar{x}$ = 29.75 (VP7 assay) and 27.85 (NS2 assay)] blood. It was decided to freeze blood samples, as they could be stored until analysed and the method was shorter.

RNA extraction kits

ANOVA of the C_T-values obtained from using the RNeasy Mini Kit [$\bar{x}$ = 29.27 (VP7 assay) and 26.88 (NS2 assay), n = 10], QIAamp RNA Blood Mini Kit [$\bar{x}$ = 30.20 (VP7 assay) and 29.22 (NS2 assay), n = 10] and QIAamp Viral RNA Mini Kit [$\bar{x}$ = 27.78 (VP7 assay) and 24.91 (NS2 assay), n = 10] showed no significant differences for the VP7 data ($p = 0.110$), but a significant difference for the NS2 data ($p = 0.002$). Multiple pairwise comparisons (LSD) identified a significant difference ($p < 0.001$) between the QIAamp RNA Blood and Viral RNA Mini kits.

A further comparison between the RNeasy Mini and QIAamp Viral RNA Mini kits was performed. A paired t-test detected no significant difference in C_T ($p > 0.104$) between the two kits [RNeasy: $\bar{x}$ = 28.79 (VP7 assay) and 27.46 (NS2 assay), QIAamp: $\bar{x}$ = 30.16 (VP7 assay) and 27.16 (NS2 assay), n = 25], yet in some instances the C_T's were up to 7.3 units lower when using the RNeasy over the QIAamp Viral kit (results not shown). In these cases, it appeared as if there was a significant inhibition of the assay when extractions were performed using the QIAamp Viral kit, especially when performing the VP7 assay and using heparinised blood. It was therefore decided to use the RNeasy Mini kit.

RNA denaturation, temperature of RT

Denaturation method accounted significantly for the variation of the slopes [$p = 0.001$ (VP7 assay) and 0.037 (NS2 assay)], but not the intercepts [$p = 0.069$ (VP7 assay) and 0.058 (NS2 assay)] of the linear regression equations. Data from the RT-runs at 42°C showed that there was a 1.3 fold increase in amount of AHSV detected when heat, instead of MMOH, was used to denature the dsRNA and the efficiency of the assay increased from 93% to 100%. The difference appeared to be greater at low virus concentrations.

Temperature of the RT significantly accounted for the variation of the slopes [$p < 0.001$ (VP7 assay) and $p = 0.503$ (NS2 assay)], but not the intercepts ($p > 0.127$) of

the linear regression equations. The significant result was due to the poor efficiency of the VP7 assay at 47°C. Results obtained from heat-denatured RNA showed that the efficiency of the VP7 assay at 42, 47 and 52°C was 100, 93 and 98% respectively; for the NS2 assay it was 90, 90 and 87%. The optimum temperature of the RT was therefore 42°C.

The addition of random hexamers to dsRNA before denaturation resulted in 18-fold higher detection of AHSV (a mean decrease in C_T of 4.16) compared to the addition of random hexamers after denaturation (paired T-test: $p = 0.001$, $n = 12$). Excluded from this test were two samples with a low level of AHSV which tested negative when random hexamers were added after denaturation.

A paired T-test detected a significant difference in C_T when dsRNA was denatured at 98°C for five min compared to 95°C for one min [$\bar{x}$ difference = 0.370, $p = 0.003$, $n = 13$ (VP7 assay) and $\bar{x}$ difference = 0.345, $p < 0.001$, $n = 13$ (NS2 assay)]. The latter resulted in lower C_T values and was therefore a more sensitive method for detecting AHSV.

Reverse transcription

One-way ANOVA showed no significant differences ($p > 0.284$) in the slopes nor the intercepts of the regression equations derived from using the different RT kits. However, the C_T-values obtained from using the Applied Biosystems and Ambion kits were on average 1.79 and 1.99 ($n = 19$) lower compared to the results obtained from the Roche kit. In addition, C_T versus log concentration plots showed that the data were more sigmoidal in shape than linear when the Roche kit was used, indicating inhibition of the RT reaction at high RNA concentrations, which was not evident in the data obtained from the other kits. Of the three kits, the Roche kit was judged to have performed the worst, while no differences between the Applied Biosystems and Ambion kits were detected. The Applied Biosystems kit was chosen based on costs.

When the RNeasy Mini Kit was used to extract RNA from washed and pelleted RBC membranes (500 µl starting volume), a mean RNA concentration of 29.8 ng/µl ($s = 22.8$, $n = 15$, elution volume = 30 µl) was measured in the eluate. With Reverse Transcription Reagents (Applied Biosystems), up to 500 ng RNA can be reverse transcribed in a 20 µl reaction. The maximum volume of RNA (7.7 ul) was therefore used in the RT reaction.

Using random hexamers instead of gene-specific primers in the RT step resulted in C_T's that were on average 0.27 (n = 12) lower and the detection limit was 100-fold lower (10^6 and 10^4-fold dilution for random hexamers and gene specific primers respectively) and was therefore a more sensitive method.

A paired T-test detected a significant difference in C_T when the length of the RT was increased from 30 min to 60 min for the VP7 assay ($\bar{x}$ difference = 0.747, $p < 0.001$, n = 13) but not for the NS2 assay ($\bar{x}$ difference = 0.081, $p = 0.285$, n = 13), suggesting a selective increase in VP7 cDNA, but not NS2 cDNA when the length of the RT was increased.

PCR kits

There were significant differences in C_T's between the High Capacity RNA-to-cDNA and the Reverse Transcription Reagents kits [$\bar{x}$ difference = 0.79, $p = 0.033$, n = 14 (VP7 assay) and $\bar{x}$ difference = 2.37, $p < 0.001$, n = 14 (NS2 assay)]. Lower C_T's were obtained in the VP7 assay with the former kit, and in the NS2 assay with the latter kit. Poor results were obtained using the NS2 assay and the High Capacity kit and the Reverse Transcription Reagents kit was therefore selected for RT.

Significant differences between the different PCR kits were detected with the VP7 assay ($p = 0.030$) but not with the NS2 assay ($p = 0.324$). There was a mean difference in C_T of 1.2 between the TaqMan® Gene Expression Master Mix and the TaqMan® Fast Universal PCR Master Mix kits, with the latter the more sensitive. Paired T-tests showed significant differences in C_T's [$\bar{x}$ difference = 0.99, $p < 0.001$, n = 14 (VP7 assay) and $\bar{x}$ difference = 0.19, $p = 0.009$, n = 15 (NS2 assay)] between the TaqMan® Universal PCR Master Mix and TaqMan® Fast Universal PCR Master Mix kits, with the latter the more sensitive kit.

No significant differences were detected between different primer concentrations in the PCR reaction [$p = 0.965$ (VP7 assay) and 0.212 (NS2 assay)]. The mean C_T's for the 900nm, 300nM and 50nM groups were 32.01, 32.16 and 32.51 (VP7 assay) and 32.16, 31.70 and 34.95 (NS2 assay) respectively (n = 8). A final primer concentration of 300nm was selected, to provide sufficient primer for the PCR reaction but on the other hand to limit the concentration in a duplex reaction.

No significant differences were detected between different probe concentrations in the PCR reaction [p = 0.739 (VP7 assay) and 0.625 (NS2 assay)]. The mean C_T's for the 250nm, 150nM and 50nM groups were 30.62, 31.18 and 32.43 (VP7 assay) and 30.57, 30.88 and 32.53 (NS2 assay) respectively (n = 8). A final probe concentration of 250nm was selected, as it resulted in the lowest C_T's, steepest slopes and highest final fluorescence of the real-time curves.

There was a significant difference in C_T-values when the VP7 assay was run as a singleplex assay compared to a duplex assay together with NS2 ($p < 0.001$); likewise for the NS2 assay ($p = 0.014$). Lower C_T-values were obtained with the singleplex assay compared to the duplex assay [$\bar{x}$ difference = 0.33 (VP7) and 0.22 (NS2), n = 15]. The significant, but slightly lower sensitivity of the duplex assay compared to the singleplex assay was balanced by the savings in time and money.

Figure S1 Amplification plot of a real-time RT-qPCR of ten-fold serial dilutions of 10^8 to 10^1 AHSV VP7 and NS2 dsRNA copies, performed in triplicate on a Step One Plus PCR machine (Applied Biosystems).

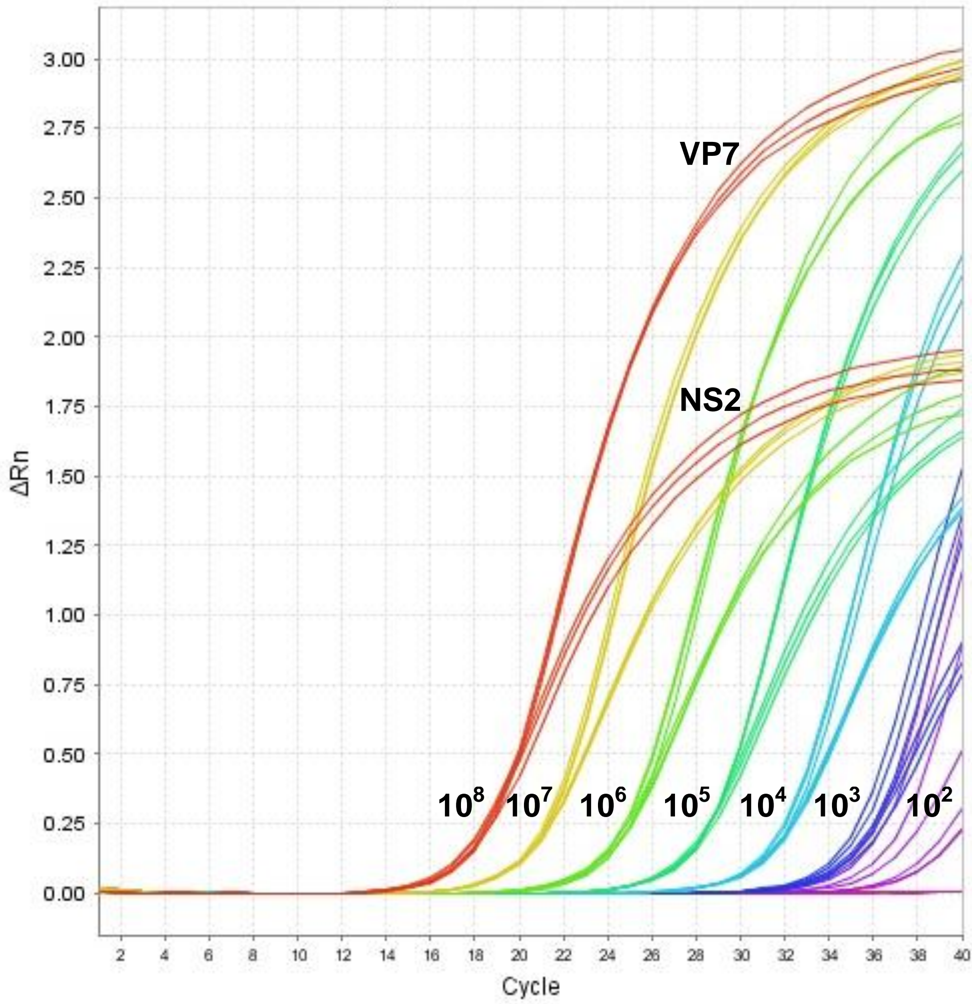

Table1

Table 1. (a) Full length RT-PCR and, **(b)** real-time RT-qPCR AHSV assay primer and probe sequences.

	Gene	Name	Sequence
(a)	VP7	SZ_AHSV_VP7F_0001_0020	5′ -GTT AAA ATT CGG TTA GGA TG
	VP7	SZ_AHSV_VP7R_1160_1179	5′ -GTA AGT GTA TTC GGT ATT GA
	NS1	MQ_AHSV_NS1F_0001_0023	5′ -GTT AAA GAA CCT AGG CGG TTT GG
	NS1	MQ_AHSV_NS1R_1728_1749	5′ -GTA AGT TTG TGA ACC AGG GGG A
	NS2	MQ_AHSV_NS2F_0001_0022	5′ -GTT TAA AAA TCC GTT CGT CAT C
	NS2	MQ_AHSV_NS2R_1150_1166	5′ -GTA TGT TGA AAT CCG CG
(b)	VP7	MQ_AHSV_VP7F_1050_1071	5′ -AGA GCT CTT GTG CTA GCA GCC T
	VP7	MQ_AHSV_VP7R_1108_1128	5′ -GAA CCG ACG CGA CAC TAA TGA
	VP7	MQ_AHSV_VP7P_1092_1106	5′FAM-TGC ACG GTC ACC GCT
	NS2	MQ_AHSV_NS2F_1045_1065	5′ -GGG AAG TGC TAC RCA TTA CCA[a]
	NS2	MQ_AHSV_NS2R_1083_1105	5′ -TGC TGG GAG AAT CAT GTA ACT CA
	NS2	MQ_AHSV_NS2P_1067_1081	5′VIC-TGC TGT GCT AAT GAC

[a] R = G/A

Table2

Table 2. Variability of AHSV VP7, NS1 and NS2 gene sequences.

African horse sickness virus	VP7 gene	NS1 gene	NS2 gene	NS3 gene[a]
Nucleotide sequences				
Number of sequences	52	51	54	145
Number of unique sequences	43	42	37	66
Nucleotide substitution model used[b]	TVM + Γ	TIM + I	GTR + I + Γ	GTR + I + Γ
Median pairwise genetic distance	0.054	0.034	0.029	0.663
and range	0.001-0.302	0.001-0.081	0.001-0.059	0.002-1.235
Protein sequences				
Number of unique sequences	22	28	23	49
Minimum pairwise protein identity (%)	97.1	97.3	95.9	64.2

[a] NS3 gene included for comparison, Quan *et al.*, 2008.
[b] Posada and Crandall, 1998.
Γ, shape parameter of the gamma distribution; GTR, general time reversible model (Rodriguez et al., 1990); I, invariable sites; TIM, transitional model; TVM, transversional model.

Table3

Table 3. Specificity of the AHSV VP7 and NS2 assays. Samples were classified as negative if no cycle threshold (C_T) was obtained after 40 cycles of the PCR.

Virus serotype (isolate reference)	C_T (VP7)	C_T (NS2)
AHSV 1 (Jane1 BHK#4 Oct 02)	13.70	13.35
AHSV 2 (Jane2 BHK#4 Oct 02)	20.30	21.30
AHSV 3 (Jane3 BHK#5 Oct 02)	17.65	18.07
AHSV 4 (Jane4 BHK#4 Sep 02)	16.97	16.96
AHSV 5 (Jane5 BHK#4 Oct 02)	14.73	15.33
AHSV 6 (Jane6 114 12/07/01 +1BHK)	16.01	15.82
AHSV 7 (Jane7 Karen 12/07/01 +1BHK)	17.81	17.33
AHSV 8 (Jane8 BHK#5 Oct 02)	19.89	19.74
AHSV 9 (Jane9 #3 E41/02 23/05/02)	16.59	16.90
BTV 1 (pp 28/01/98)	Negative	Negative
BTV 2 (pp 28/01/98)	Negative	Negative
BTV 3 (pp 28/01/98)	Negative	Negative
BTV 4 (plaque A 13/03/98)	Negative	Negative
BTV 5 (26/06/98)	Negative	Negative
BTV 6 (26/06/98)	Negative	Negative
BTV 7 (26/06/98)	Negative	Negative
BTV 8 (09/07/98)	Negative	Negative
BTV 9 (pp 21/11/97)	Negative	Negative
BTV 10 (pp 21/11/97)	Negative	Negative
BTV 11 (pp 09/07/98)	Negative	Negative
BTV 12 (pp 09/07/98)	Negative	Negative
BTV 13 (pp 20/07/98)	Negative	Negative
BTV 14 (pp 20/07/98)	Negative	Negative
BTV 15 (pp 20/07/98)	Negative	Negative
BTV 16 (pp 09/02/98)	Negative	Negative
BTV 17 (pp 09/02/98)	Negative	Negative
BTV 18 (pp 10/02/98)	Negative	Negative
BTV 19 (pp 10/02/98)	Negative	Negative
BTV 20 (pp 16/02/98)	Negative	Negative
BTV 21 (pp 16/02/98)	Negative	Negative
BTV 22 (plaque B 08/10/98)	Negative	Negative
BTV 23 (pp 17/02/98)	Negative	Negative
BTV 24 (13/03/98)	Negative	Negative
EEV 1 (Bryanston +1BHK 26/11/04)	Negative	Negative
EEV 2 (Cascara #3BHK 09/09/02)	Negative	Negative
EEV 3 (Gamil 06/08/98)	Negative	Negative
EEV 4 (Kaalplaas #2Vero 01/10/96)	Negative	Negative
EEV 5 (Kyalami #3Vero 2BHK 28/01/02)	Negative	Negative
EEV 6 (Potchefstroon 25/02/02)	Negative	Negative
EEV 7 (E21/20 #6BHK 23/05/03)	Negative	Negative
EHDV (83/132A 21/11/97)	Negative	Negative
PALV (5 #2Vero 26/02/93)	Negative	Negative

AHSV, African horse sickness virus; BTV, bluetongue virus; EEV, equine encephalosis virus; EHDV, epizootic hemorrhagic disease virus; PALV, Palyam virus.

Figure1

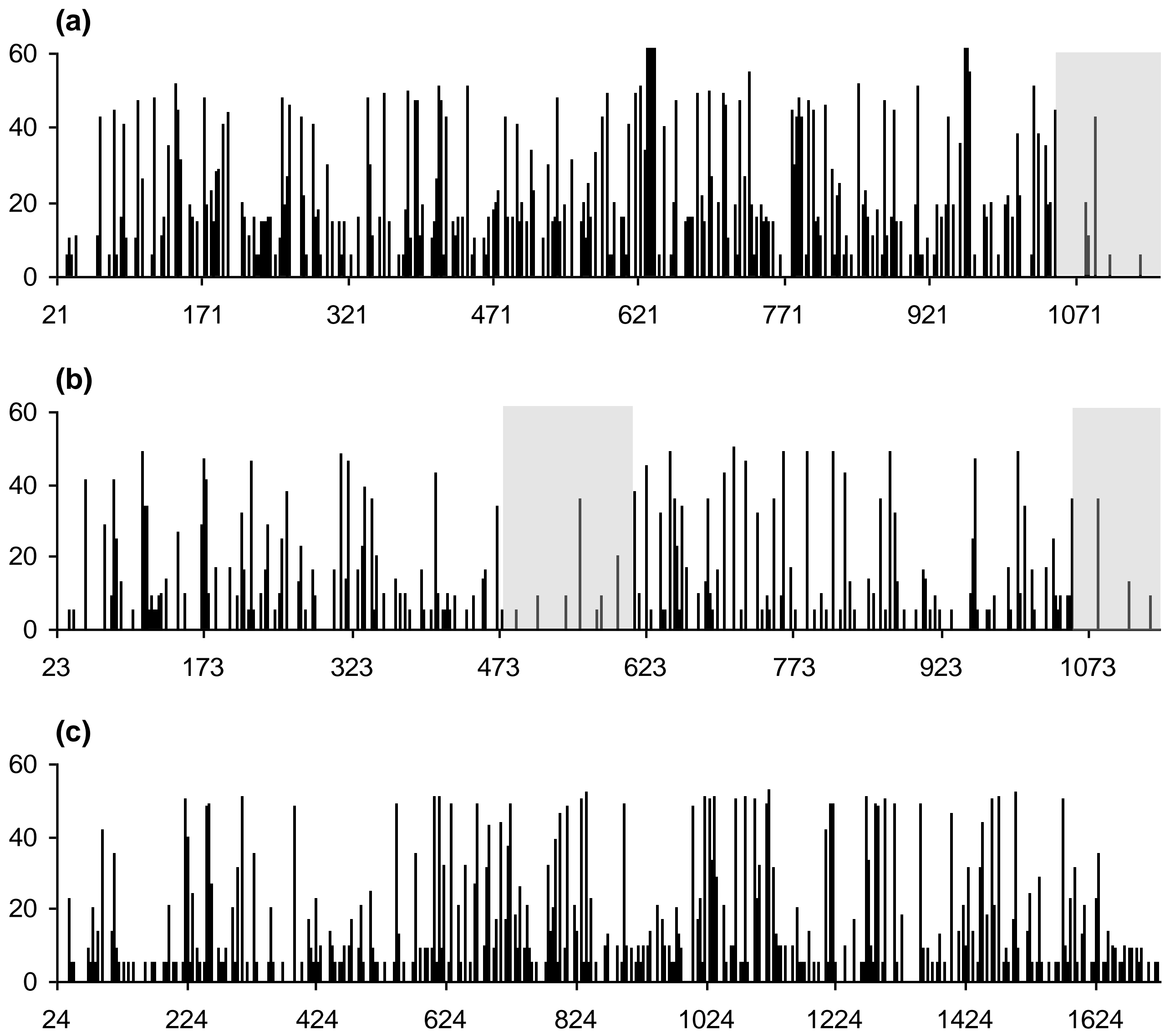

Figure2

(a) VP7 - Moulay *et al.*, 1995; Sailleau *et al.*, 1997; Zientara *et al.*, 1993, 1994, 1995b and 1998 (full length)

```
                      10        20   1160      1170
                  ...|....|....|....|  |....|....|....|....
Primer template   gttaaaattcggttaggatg  tcaataccgaatacacttac
NC_006011* (4)    ....................  ....................
AM883171          ...t................  ................c...
FJ011114          ...t................  .........-......c...
AF545433          ................c...  ....................
U90337            ...t................  ....................
```

(b) VP7 - Moulay *et al.*, 1995; Zientara *et al.*, 1995b (nested)

```
                         30        40   270       280
                  ..|....|....|....|.  ...|....|....|....|.
Primer template   cgcgatagcagcaagagcc  tatgatcaggcgttggcaac
NC_006011* (40)   ...................  ....................
U90337            .......cg..........  ........a...........
E07305_VP7 (2)    .........g.........  ....................
E12505_VP7 (11)   ...................  .....c..........g..
E10205_VP7        ............g......  .....c..........g..
E03505_VP7 (2)    ..................t  .....c..........g..
E16004_VP7        ...................  ........a..a........
Jane8_VP7 (3)     ...................  ........a...........
```

(c) VP7 - Agüero *et al.*, 2008

```
                        1040      1050          1070           1100      1110
                  ...|....|....|....|.  ....|....|....|...  |....|....|....|....
Primer template   ccagtaggccagatcaacag  gctagcagcctaccacta  acggtcaccgctttcattag
NC_006011* (21)   ....................  ..................  ....................
Jane9_VP7 (4)     ..............t.....  ..................  ....................
Jane3_VP7 (13)    ........t...........  ..................  ....................
E04404_VP7        ........t...........  ..................  ............c.......
E05805_VP7 (2)    ...........a........  ..................  ....................
E09605_VP7        ........t..a........  ..................  ....................
E12505_VP7 (11)   ..g..............t..  ..................  ....................
E00605_VP7 (6)    .................t..  ..................  ....................
E06105_VP7        ..g........a.....t..  ..................  ....................
```

(d) VP7 - Fernández-Pinero *et al.*, 2009

```
                      820       830        860       870         900       910
                  |....|....|....|....|.  ..|....|....|...  ....|....|....|....|.
Primer template   ggctccaacactcacaagatgt  tggcacgccttacgcgc  tatgcagcctattaatccgcc
NC_006011* (27)   .........g............  ........a........  .....................
Jane7_VP7         .........g............  ........a........  ............c........
E06405_VP7        ......g..g............  ........g........  ............c..c.....
E23904_VP7        ......g..g............  ........a........  ......a.....c........
Jane3_VP7         ......g..g............  ........a........  ............c........
E13005_VP7 (2)    ......g..g..g.........  ........a........  ............c........
E04404_VP7        ......g..g..g.........  ........a........  .........c..c........
E14405_VP7 (17)   .........g............  ........a........  ............c........
E05805_VP7        .........g.......a....  ........a........  ............c........
E07305_VP7 (2)    ......................  .....t..a........  ............c........
E11005_VP7        ........tg............  ........a........  .....................
E03505_VP7        .........g...........c  ........a........  ............c........
E14904_VP7        .........g............  ........a........  ............c........
E16004_VP7        ......g..g...........c  ........a.....t..  ..................a..
Jane8_VP7 (3)     ............t.........  .........c.......  .........a...........
```

(e) VP7 - This study

```
                  1050      1060      1070        1100       1110      1120
                  |....|....|....|....|.  ...|....|....|.  ..|....|....|....|...
Primer template   agagctcttgtgctagcagcct  tgcacggtcaccgct  tcattagtgtcgcgtcggttc
NC_006011* (60)   ......................  ...............  .....................
```

(f) NS2 - Stone-Marschat *et al.*, 1994

```
                    180       190      580       590
                  ....|....|....|....|  |....|....|....|....
Primer template   accccgattccaaaaagtta  ctgtagaatgtatgggggag
NC_006016* (47)   ....................  ....................
Jane9_NS2 (4)     ........c...........  ....................
Jane8_NS2 (3)     ....................  ...............a....
E23904_NS2 (2)    ..t.................  ....................
E16004_NS2 (2)    ....................  ...............a....
```

(g) NS2 - Bremer and Viljoen, 1998

```
                  700       710        920
                  .|....|....|...  |....|....|....
Primer template   tggcacgaagacatg  cattggcgatgagag
NC_006016* (14)   ...............  ...............
Jane9_NS2         ...............  .......t.......
Jane3_NS2 (17)    ....g..........  ...............
E12505_NS2 (23)   ....g........c.  ...............
E09104_NS2        ...tg..........  ...............
E16004_NS2        ...............  .gc............
E14904_NS2        ...............  .g.............
```

(h) NS2 - This study

```
                    1050      1060        1070      1080        1090      1100
                  |....|....|....|....|  ...|....|....|.  ..|....|....|....|....|
Primer template   gggaagtgctacrcattacca  tgctgtgctaatgac  tgagttacatgattctcccagca
NC_006016* (2)    .......cg...g........  ...............  .......................
Jane9_NS2 (10)    ............g........  ...............  .......................
Jane8_NS2 (44)    ............a........  ...............  .......................
E16004_NS2 (2)    c...........a........  ...............  .......................
```

(i) NS1 - Rodriguez-Sanchez *et al.*, 2008

```
                  1060      1070               1090      1100            1250      1260
                  .|....|....|....|....  .|....|....|....|....|...  ....|....|....|....|...
Primer template   gttgacctcgctctgcttgac  tcccggtggttgattcaaaagttgc  gtcatacaacgaaggaagcgatt
NC_006020         .....................  .........................  .......................
FJ011111* (20)    .....................  ....a...............a....  .......................
E14405_NS1        .................c...  ....a...............a.c..  .......................
E13005_NS1 (4)    .....................  ....a...............a.c..  .......................
E00605_NS1 (2)    ..c........a.........  ....a...............a.c..  .......g...............
E16004_NS1 (2)    .....t.....a.........  ....a...............a.c..  .......g...............
E07105_NS1 (5)    .....................  ....a..............ga....  .......................
E12505_NS1 (22)   ............a........  .t..a........c......a....  .......................
E12005_NS1 (3)    ............a........  .t..a...............a....  .......................
Jane3_NS1         ....................t  ....a..............ga....  ......................c
D11390            ............a........  ....a....................  .......................
EU303167          .....................  ...................ga....  .......................
EU303162          ...........a.........  ....a...............a.c..  .......g...............
EU303168 (2)      ...........a.........  ....a...............a....  .......................
Jane7_NS1         ...........at........  ....a...............a....  .......................
Jane6_NS1         .....................  ....a...............a....  ................g......
```

(j) NS1 - Mizukoshi *et al.*, 1994

```
                      1210      1220   1510      1520
                  ...|....|....|....|.  |....|....|....|....
Primer template   tgaagtcttcaggaagatcg  ggatggaagagatttgactg
NC_006020         ....................  ....................
FJ011111*         ....................  ..................c.
AM883168 (20)     ....................  ....................
Jane2_NS1 (8)     ..................t.  ....................
E14405_NS1 (5)    ..................t.  ..................c.
E16004_NS1 (2)    ......t...........t.  ....................
E07105_NS1 (2)    ......t.............  ....................
E12505_NS1 (14)   ...............a..t.  ....................
E10205_NS1 (8)    ......t........a..t.  ....................
E06405_NS1        ..................t.  ...............a....
EU303168 (3)      ...............a....  ....................
Jane6_NS1         .........t..........  ............c.......
U01069 (2)        ......t.............  a...........c.......
```

Figure3
Click here to download high resolution image

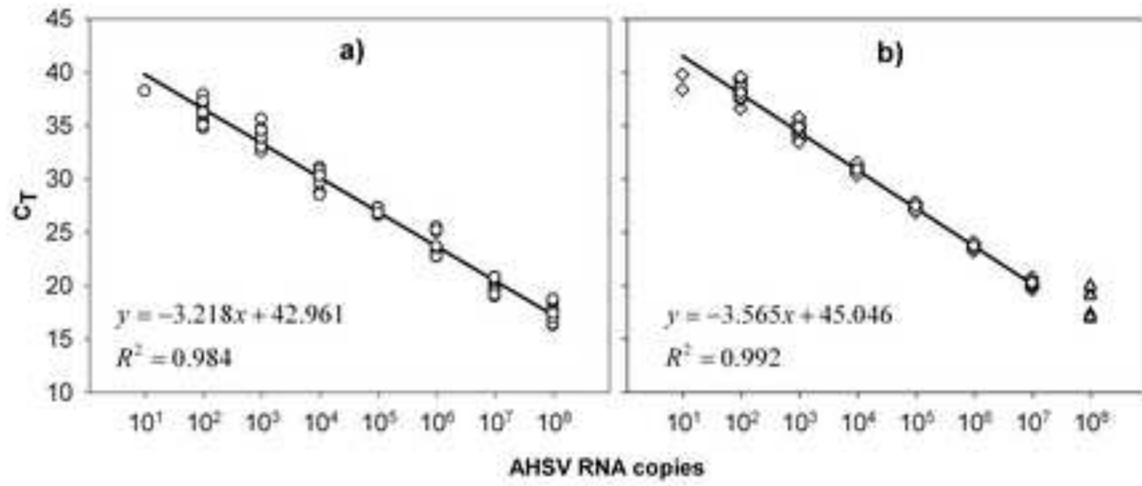

Figure4

Click here to download high resolution image

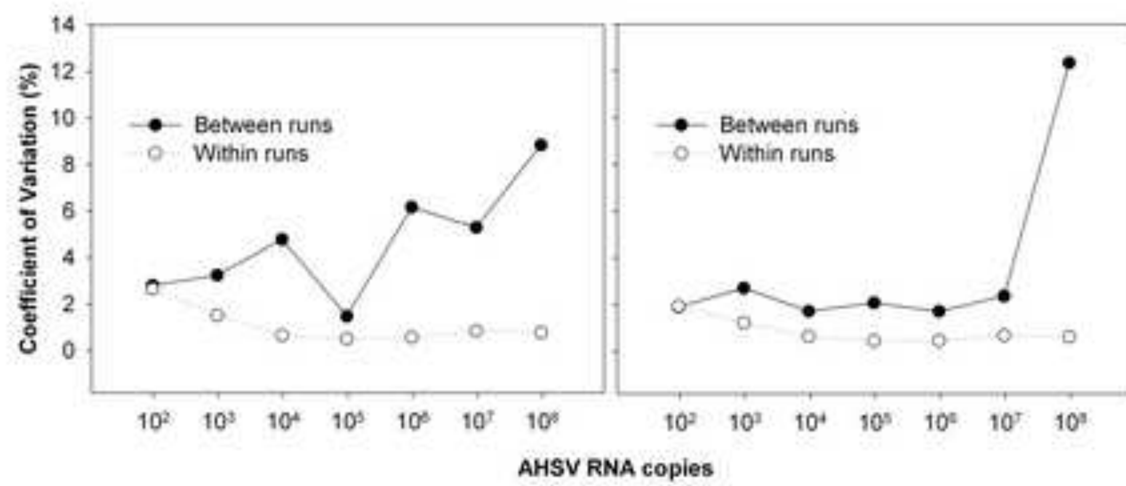

Figure5
Click here to download high resolution image

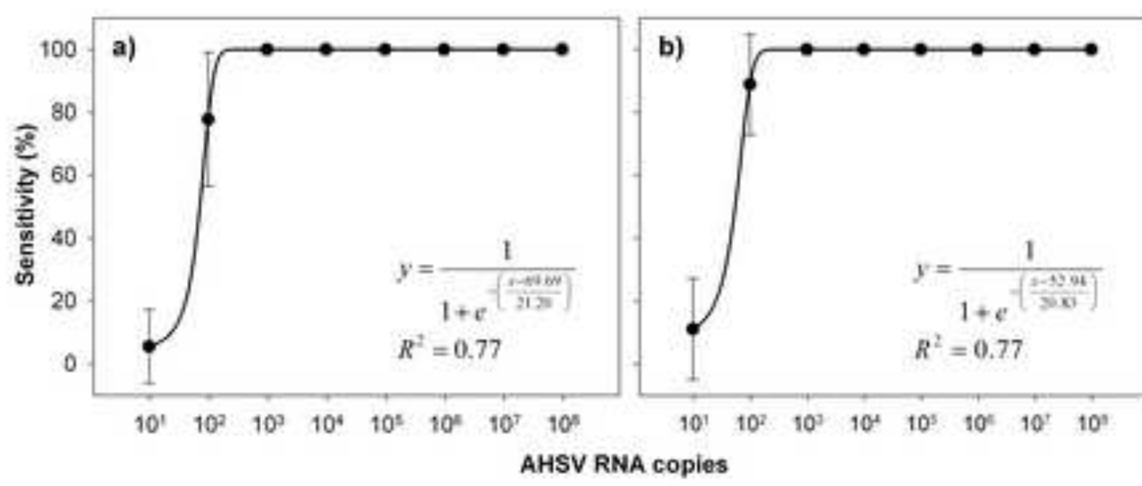

Figure6
Click here to download high resolution image