Development of a real time polymerase chain reaction assay for equine encephalosis virus

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Abstract

Equine encephalosis virus (EEV) is the cause of equine encephalosis. The disease is similar to mild forms of African horse sickness (AHS) and the two diseases are easily confused. Laboratory identification and serotyping of EEV is based on viral isolation in BHK-21 cells and a viral plaque inhibition neutralization test. These procedures are time-consuming and therefore a more rapid diagnostic assay for EEV that can distinguish EEV from African horse sickness virus (AHSV) infections was developed.

The S7 (VP7) gene from 38 EEV isolates representing all seven serotypes was amplified and sequenced. A conserved region at the 5' end of the gene was identified and used to design group-specific EEV primers and a TaqMan® MGB™ hydrolysis probe.

The efficiency of the EEV real-time RT-PCR assay was 81 %. The assay was specific, as it did not detect any of the nine serotypes of AHSV, nor 24 serotypes of bluetongue virus (BTV) and sensitive, with a 95 % limit of detection of $10^{2.9}$ TCID$_{50}$/ml blood (95 % confidence interval: $10^{2.7} - 10^{3.3}$).

The real-time format was selected because of its convenience, sensitivity and ability to produce results rapidly.

Highlights

- **The S7 genes of 38 EEV isolates were sequenced.**
- **A real-time RT-PCR assay for equine encephalosis virus was developed.**
- **The 95% limit of detection of the EEV assay was $10^{2.9}$ TCID$_{50}$.**
- **The assay was specific for EEV and did not detect other orbiviruses (BTV, AHSV).**

1. Introduction

Equine encephalosis was first described by Sir Arnold Theiler, who described a fever in horses that simulated African horse sickness (AHS), which he called "ephemeral fever" (Theiler, 1910). Theiler differentiated the disease from AHS on clinical signs (incubation period and temperature characteristics) and transmission experiments. Equine encephalosis virus (EEV) was isolated in 1967 from a Thoroughbred mare named Cascara from the Kimberley district of South Africa. Clinical signs of the affected horse included listlessness, tightening of the muscles of the face, a high temperature and an elevated pulse rate about 24 h before death. The virus was also recovered from blood samples taken from other horses on the same farm which had exhibited no clinical signs except a febrile reaction (Erasmus et al., 1970).

Equine encephalosis is endemic to southern Africa (Barnard, 1997; Venter et al., 1999) and the seroprevalence is more than 75 % in horses and in donkeys 85% (Venter et al.. 1999). In Thoroughbred horses the seroprevalence of neutralizing antibodies against one or more serotypes of the EEV was 56.9 % (Howell et al., 2002). Antibodies against EEV have been demonstrated in zebra and African elephant (Barnard, 1997; Williams et al., 1993).

EEV infection in horses was been reported recently in Israel and involved approximately 150 cases with no reported mortalities (Aharonson-Raz et al., 2011; Mildenberg et al., 2009). Circulation of EEV in Ethiopia, Ghana and The Gambia has also been reported recently (Oura et al., 2012).

EEV is transmitted between equid hosts by the bites of Culicoides spp. midges (Diptera: Ceratopogonidae), specifically C. imicola, which is regarded as the main vector of EEV (Paweska et al., 1999; Venter et al., 1999). C. imicola is the most abundant vector of the Culicoides species associated with livestock in the summer rainfall region of southern Africa. The first isolation of an EEV strain from Culicoides species in South Africa was done by Theodoridis et al. (1979). Since then, C. bolitinos has also been confirmed as a vector for EEV (Paweska and Venter, 2004).

EEV is a member of genus Orbivirus in the family Reoviridae, subfamily Sedoreovirinae consisting of species such as AHSV, bluetongue virus (BTV), and epizootic hemorrhagic disease virus (EHDV) with similar structural morphology and functional properties. The genome of EEV is similar to those of other orbiviruses and consists of ten double-stranded (ds) RNA segments encapsulated by a double-layered icosahedral shell. Each of the segments codes for a viral protein, namely seven structural proteins (VP1 to VP7) and non- structural proteins (NS1, NS2, NS3/NS3a, NS4) (Belhouchet et al., 2011; Firth, 2008; Mertens et al., 1984; Ratinier et al., 2011).

There are seven serotypes of EEV. These are EEV-1 (Bryanston), EEV-2 (Cascara), EEV-3 (Gamil), EEV-4 (Kaalplaas), EEV-5 (Kyalami), EEV-6 (Potchefstroom), and EEV-7 (E21/20) (Gorman et al., 1983; Howell et al., 2002).

Most EEV infections are subclinical in nature and mild forms are confused easily with mild forms of African horse sickness virus (AHSV) infections, as both infections exhibit similar clinical signs (Howell et al., 2004) . This makes diagnosis difficult and laboratory tests are needed to differentiate the diseases. There are various laboratory methods used in the diagnosis of EEV infection. Isolation of the virus is performed in baby hamster kidney (BHK) cells, suckling mice brains, or embryonated hen's eggs (Erasmus et al., 1970). The virus is serotyped by the plaque inhibition neutralization assay (Quan et al., 2008). A serological group-specific, indirect sandwich enzyme-linked immunosorbent assay (ELISA) is available for the detection of EEV antigen (Crafford et al., 2003). Tests for antibody detection include complement fixation (CF), agar gel immunodiffusion (AGID) or indirect immunofluorescent antibody (IFA) tests (Howell et al., 2004). The disadvantages of these methods are that they are time-consuming and only provide a retrospective diagnosis.

To date, no reverse transcription polymerase chain reaction (RT-PCR)-based assay for the detection of EEV nucleic acid has been described. Real-time RT-PCR provides several advantages over the use of conventional PCR and ELISA, including rapid turn-around with high analytical specificity, sensitivity and a reduced risk for contamination. As the clinical signs of AHSV and EEV infections in equines may be difficult to distinguish a rapid and reliable diagnostic real-time RT-PCR assay for EEV is needed to for rapid diagnosis of this infection.

This paper describes the development and optimisation of a real-time RT-PCR assay for the sensitive and specific detection of EEV in samples from horses infected naturally with EEV. This was accomplished by sequencing the S7 (VP7) gene of 38 EEV isolates representing all seven serotypes and identifying a conserved region for the design of an EEV real-time RT-PCR assay using a TaqMan® MGB™ hydrolysis probe. Critical control parameters of the assay, as well as the repeatability, analytical sensitivity and specificity of the assay were estimated.

2. Materials and methods

2.1 Development of EEV real-time RT-PCR assay

An EEV S7 (VP7) gene sequence (FJ183391) obtained from GenBank® (www.ncbi.nlm.nih.gov/genbank) was used with FastPCR software V6.1.47 (Kalendar et al., 2009) to design terminal primers for amplification and sequencing of the EEV S7 gene.

EEV isolates representing all seven recognized serotypes of EEV were sequences (Table 1).

Viral dsRNA was extracted from EEV cell culture isolates. The contents of a flask were agitated and 500 μl transferred to a 1.5 ml eppendorf tube. Samples were spun at 11 000 g for 5 min in a 5417C centrifuge (Eppendorf). The supernatant was discarded and the cell pellet mixed with 50 μl of phosphate buffered saline (PBS). Total nucleic acid extractions from the cell pellet were performed using a MagMax™-96 Total RNA Isolation kit (Lifetech), according to the manufacturer's instructions. The samples were processed in a MagMax™ Express Particle Processor (Lifetech) and a custom

Table 1 : EEV isolates used for sequencing of the S7 (VP7) gene.

Name	Serotype	Passage number	Isolation date	GenBank
Reference strain	1 (Bryanston)	1	2004/11/26	KF483827
E080099	1 (Bryanston)	6	2008/07/03	KF483807
E080243	1 (Bryanston)	6	2008/08/14	KF483814
E080340_1	1 (Bryanston)	3	2009/02/19	KF483816
E080341_2	1 (Bryanston)	3	2009/02/19	KF483817
E080342	1 (Bryanston)	3	2009/02/12	KF483818
E090039	1 (Bryanston)	4	2009/04/03	KF483823
E090047	1 (Bryanston)	3	2009/04/03	KF483798
E090059	1 (Bryanston)	1	2009/04/09	KF483824
Reference strain	2 (Cascara)	3	2002/09/09	KF483795
E100043	2 (Cascara)	-	2010/05/06	KF483796
Reference strain	3 (Gamil)	-	1998/08/06	KF483828
Reference strain	4 (Kaaplaas)	-	1998/08/06	KF483829
E080010_5	4 (Kaaplaas)	2	2008/01/31	KF483800
E080013_2	4 (Kaaplaas)	2	2008/02/07	KF483801
E080067	4 (Kaaplaas)	2	2008/03/20	KF483804
E080129	4 (Kaaplaas)	2	2008/04/10	KF483799
E080210_3	4 (Kaaplaas)	-	-	KF483810
E080229_2	4 (Kaaplaas)	-	-	KF483812
E080229_5	4 (Kaaplaas)	3	2008/05/29	KF483813
E080260_10	4 (Kaaplaas)	4	2008/07/17	KF483815
E100058	4 (Kaaplaas)	3	2010/05/06	KF483826
Reference strain	5 (Kyalami)	3	2002/01/28	KF483830
E090010_6	5 (Kyalami)	5	2009/04/09	KF483819
E090010_15	5 (Kyalami)	3	2009/03/05	KF483820
E090010_19	5 (Kyalami)	3	2009/03/05	KF483821
E090011	5 (Kyalami)	4	2009/03/05	KF483822
Reference strain	6 (Potchefstroom)	-	2002/02/25	KF483831
E100020	6 (Potchefstroom)	4	2010/04/15	KF483825
Reference strain	7 (E21/20)	6	2003/05/23	KF483832
E080026	7 (E21/20)	2	2008/02/21	KF483802
E080038	7 (E21/20)	2	2009/03/07	KF483803
E080070	7 (E21/20)	7	2008/11/27	KF483805
E080089_3	7 (E21/20)	-	2008/07/31	KF483806
E080146	7 (E21/20)	9	2008/10/02	KF483808
E080181	7 (E21/20)	-	-	KF483809
E080186_1	7 (E21/20)	8	2008/08/07	KF483797
E080207	7 (E21/20)	-	-	KF483811

- unknown passage number or isolation date.

protocol (Supplementary data) run before elution of the RNA in 50 µl Elution Buffer. The RNA was stored at -20 °C until used.

Extracted viral nucleic acid was denatured with 0.2 M of methyl mercury (II) hydroxide (MMOH) and amplified with a one-step RT-PCR as described previously (Quan et al., 2008). The EEV S7 gene was amplified in two overlapping sections using primer EEV_VP7_F0007_0027 (ttt ggc caa caa gat gga tgc) with primer EEV_VP7_R0588_0609 (ctc gtg tac att gca aaa ggt c), and primer EEV_VP7_F0495_0516 (ttc agg tga gcc tta cgc cga a) with primer EEV_VP7_R1151_1175 (gta aca cgt ttg gcc tca gac gtt t). An annealing temperature of 55 °C was used and the PCR products visualized on a 1.5 % agarose gel prepared with TAE buffer.

ExoSAP-IT (Affymetrix) was used according to the manufacturer's instructions to purify the PCR products. Sequencing reactions were prepared using a BigDye Terminator v3.1 cycle sequencing kit (Lifetech). Reactions consisted of 2 µl Ready Reaction Premix, 1 µl of BigDye Sequencing Buffer, 3.2 pmol primer and 5 µl PCR products made up to 10 µl in H2O. A standard sequencing protocol and sequencing product purification method using ethanol/NaOAc/EDTA precipitation (Lifetech) was followed. Samples were analysed with an ABI 3130xl Genetic Analyzer (Lifetech) using POP-7 polymer and a 36 cm capillary.

The Staden software package v1.5 (Staden, 1996; Staden et al., 2000) was used for sequence assembly, ClustalX v2.0.5 (Larkin et al., 2007) to align sequences and BioEdit Sequence Alignment Editor v7.0.9 software (Hall, 1999) to edit sequences. Conserved regions within the S7 gene were identified and used to design a real-time RT-PCR assay with a TaqMan® minor groove binder (MGB™) hydrolysis probe, using Primer Express 3.0 software (Lifetech) and FastPCR software v6.1.47 (Kalendar et al., 2009).

2.2. EEV real-time RT-PCR assay

The nucleic acid from 50 µl of blood was extracted using a MagMax™-96 Total RNA Isolation kit (Lifetech) according to the manufacturer's instructions, and a MagMax™ Express Particle Processor (Lifetech). Nucleic acid was eluted in 50 µl Elution Buffer.

Five µl RNA were added to 1 µl 25X primer/probe mixture (400 nM/120 nM final concentrations) and 4 µl nuclease-free water. The probe was labeled with NED™ (Lifetech). The mixture was denatured by heating at 95 °C for 1 min on a heatblock, and then cooled quickly on ice. VetMAX™-Plus One-Step RT-PCR kit (Lifetech) reagents were added (12.5 µl 2X RT-PCR buffer, 1.5 µl nuclease free water and 1 µl enzyme). The assay was performed following the manufacturer's recommendations on a StepOnePlus™ Real-Time PCR System (Lifetech). Samples were classified as positive if the normalised fluorescence for the EEV assay exceeded a 0.1 threshold.

2.3. Assay characteristics

The assay was tested on tissue culture reference isolates of 24 serotypes of BTV, nine serotypes of AHSV and seven serotypes of EEV to determine the analytical specificity.

The efficiency of the assay was calculated using a ten-fold dilution series of blood (negative for EEV by virus isolation and RT-PCR and obtained from a clinically normal horse) spiked with EEV-2 obtained from tissue culture isolation (E118/12, $10^{7.9}$ TCID$_{50}$/ml). The dilution series was tested five

times in a single run. PCR efficiency were determined by the formula: PCR efficiency (%) = 100 × $(10^{1/slope} -1)$.

A two-fold dilution series, consisting of six separate dilutions of EEV-spiked blood, was made to cover the non-linear range of the assay. Each dilution was extracted five times and each extract tested in five independent runs. The results of these analyses were used to calculate the 95 % limit of detection (LOD) (input concentration giving a positive result in 95 % of the replicates) by probit analysis.

The inter-run, intra-run and total standard deviations (SD) were calculated by the formulas: inter-run SD, standard deviation of the means of all runs; intra-run SD, mean of the standard deviations of all runs; total SD, standard deviation of all replicates. The total coefficient of variation (CV) was calculated by the formula: total CV = total SD/(mean cycle threshold (C_T)-value of all replicates).

3. Results

3.1. Primer design

The EEV S7 genes of isolates all 7 serotypes of EEV was amplified in two overlapping segments 581 bp and 656 bp in length (Table 1).

The minimum percentage identity between isolates was 92.6 % (82 differences out of 1123 nucleotides). These differences occurred between the reference EEV-6 (Potchefstroom) strain, and EEV-5/EEV-7 isolates.

Conserved regions within the EEV S7 gene were identified (Figure 1a) and a 78 nucleotide TaqMan® MGB™ real-time RT-PCR developed at the 5'-end of the S7 gene (Figure 1b Table 2 and supplementary data). There was 100 % identity of the forward primer and TaqMan® MGB™ hydrolysis probe with all the EEV S7 sequences. A redundant reverse primer was designed as there was one nucleotide mismatch between the primer and the EEV S7 sequences. A BLAST analysis of the primers and probe showed specificity for EEV sequences.

An alignment of EEV, epizootic hemmorhagic disease virus (EHDV), BTV and AHSV S7 sequences showed very little identity between the viruses. Using FJ183391 as a reference, there was maximum identity of 72 % over a 16 % region of the EHDV S7 gene, 66% over a 18 % region of the BTV S7 gene, and 66% over a 28% region of the AHSV S7gene.

3.2. Assay characteristics

The EEV real-time RT-PCR assay was specific, detecting all seven serotypes of EEV, but not AHSV nor BTV (Table 3).

The assay was linear in the range tested, i.e. $10^{2.9}$ - $10^{6.9}$ TCID$_{50}$/ml blood. The efficiency of the assay was calculated to be 81 % (Figure 2a).

The assay was sensitive, with a 95 % LOD determined by probit analysis of $10^{2.9}$ TCID$_{50}$/ml blood (95 % confidence interval: $10^{2.7}$-$10^{3.3}$) (Figure 2b). This corresponded a C_T of 38.42.

Table 2 : Real-time RT-PCR assay targeting the S7 (VP7) gene of EEV.

Name	Type	Position	Sequence (5'-3')
EEV_VP7_F0028_0048	Forward primer	28-48	GAT AGC GGC TAG AGC CCT TTC
EEV_VP7_P0054_0072	MGB™ probe	54-72	TAA GAG CAT GTG TTA CTG C
EEV_VP7_R0085_0106	Reverse primer	85-106	AAC TTG AGG AGC CAT R*GT AGC T

*R=A/G.

Table 3 : Results of an EEV S7 (VP7) real-time RT-PCR assay tested on various reference orbiviruses. Samples were classified as negative if no fluoresence above the threshold (0.1) was detected within 35 cycles.

Virus	Serotype	C_T-value	Passage number	Cell line	Date isolated
AHSV	1	Negative	4	BHK	2002/10
AHSV	2	Negative	4	BHK	2002/10
AHSV	3	Negative	5	BHK	2002/10
AHSV	4	Negative	4	BHK	2002/09
AHSV	5	Negative	4	BHK	2002/10
AHSV	6	Negative	3	BHK	2002/08
AHSV	7	Negative	5	BHK	2003/03/14
AHSV	8	Negative	5	BHK	2002/10
AHSV	9	Negative	5	BHK	2002/05/23
BTV	1	Negative	-	-	1998/01/28
BTV	2	Negative	-	-	1998/01/28
BTV	3	Negative	-	-	1998/01/28
BTV	4	Negative	-	-	1998/03/13
BTV	5	Negative	-	-	1998/01/26
BTV	6	Negative	-	-	1998/01/26
BTV	7	Negative	-	-	1998/01/26
BTV	8	Negative	-	-	1998/07/09
BTV	9	Negative	-	-	1997/11/21
BTV	10	Negative	-	-	1997/11/21
BTV	11	Negative	-	-	1998/07/09
BTV	12	Negative	-	-	1998/07/09
BTV	13	Negative	-	-	1998/07/20
BTV	14	Negative	-	-	1998/07/20
BTV	15	Negative	-	-	1998/07/20
BTV	16	Negative	-	-	1998/02/09
BTV	17	Negative	-	-	1998/02/09
BTV	18	Negative	-	-	1998/02/10
BTV	19	Negative	-	-	1998/02/10
BTV	20	Negative	-	-	1998/02/16
BTV	21	Negative	-	-	1998/02/16
BTV	22	Negative	-	-	1998/10/08
BTV	23	Negative	-	-	1998/02/17
BTV	24	Negative	-	-	1998/03/13
EEV	1	21.57	1	BHK	2004/11/26
EEV	2	21.54	3	BHK	2002/09/09
EEV	3	21.07	-	-	1998/08/06
EEV	4	19.64	-	-	1998/08/06
EEV	5	21.28	2	BHK	2002/01/28
EEV	6	20.79	-	-	2002/02/25
EEV	7	22.59	6	BHK	2003/05/23

AHSV - African horse sickness virus, BHK – baby hamster kidney, BTV - bluetongue virus, C_T - cycle threshold, EEV - equine encephalosis virus, dash indicates unknown.

Figure 1a
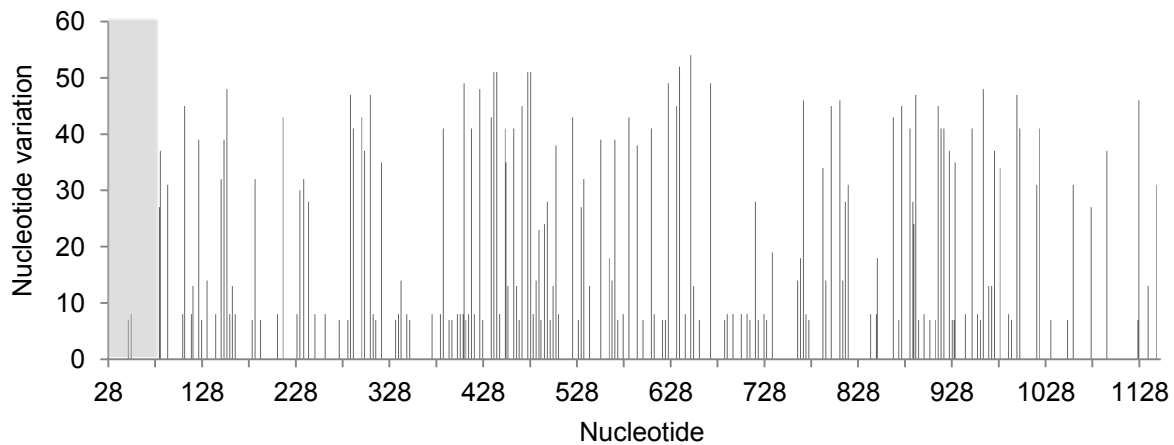
Figure 1b
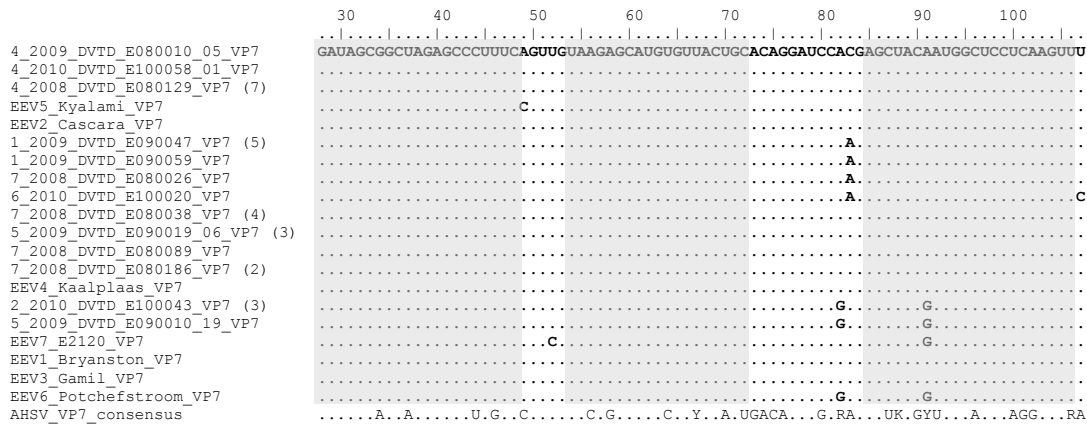
Fig. 1. (a) Plot of conservation sites on the VP7 gene, calculated using quality scores in ClustalX. No column at a nucleotide position indicates 100% conservation, the higher the column, the greater the nucleotide variation at that site. Genbank accession number FJ183391 was used for nucleotide numbering. The conserved region where the assay was developed is indicated by a grey block. (b) Detail of the grey block in 1a. EEV S7 (VP7) gene sequences and an African horse sickness virus (AHSV) S7 (VP7) consensus sequence (Quan et al., 2010) included for comparison. Dots indicate identity and letters differences with the first sequence in the group. Identical sequences are represented only once in the figure and the number of identical sequences are indicated in brackets after the name of a random sequence in the group. The location of the real-time RT-PCR assay primers and probe are indicated by grey shaded boxes.

9
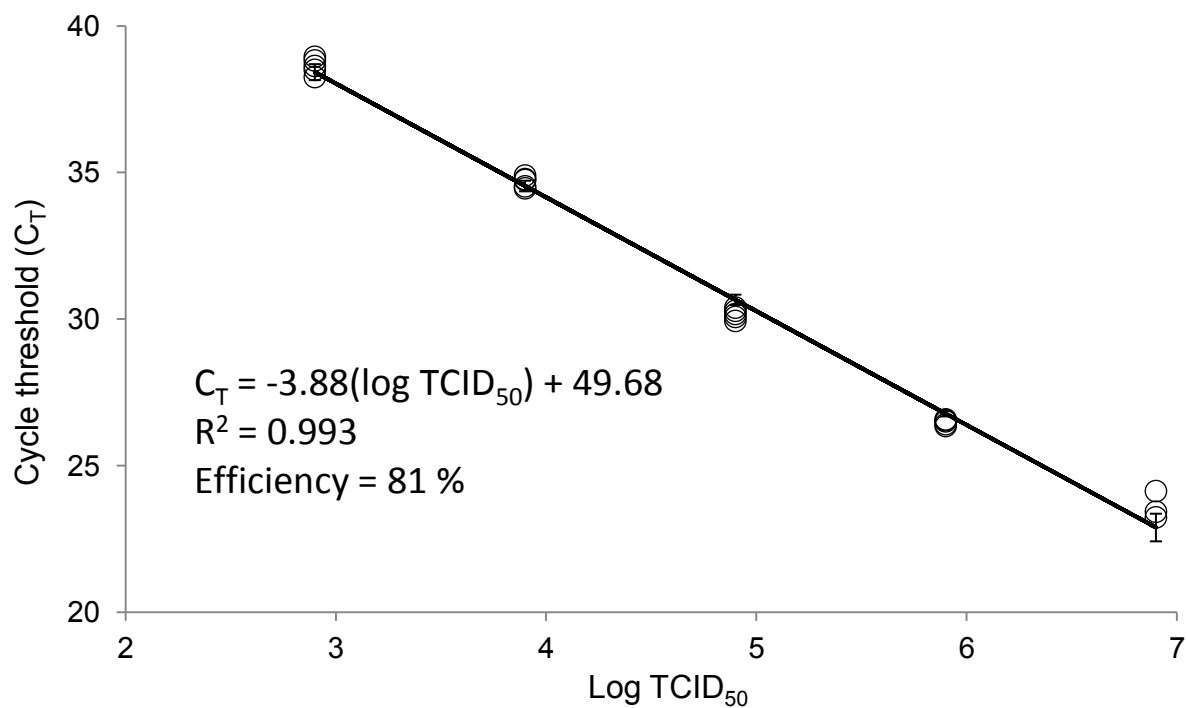
Figure 2a

Figure 2b
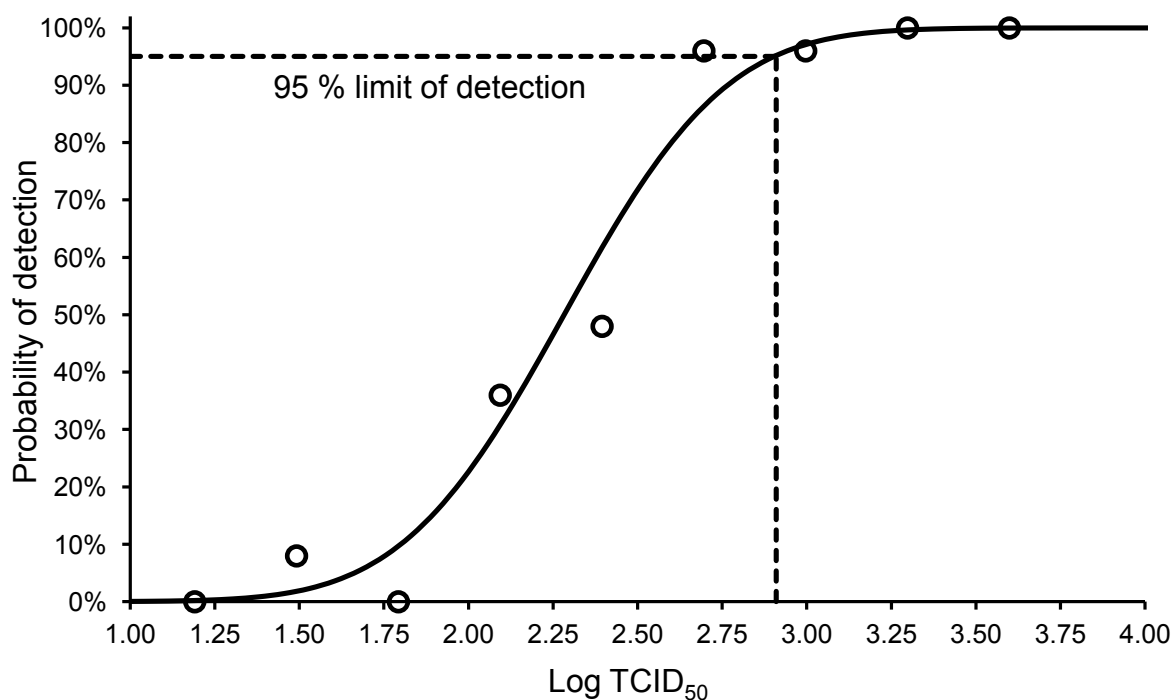
Fig. 2. (a) Regression ± standard deviation of a ten-fold dilution series of blood spiked with EEV obtained from tissue culture isolation. Each dilution was repeated five times in a run. (b) A two-fold dilution series of EEV-spiked blood covering the non-linear range of the EEV assay. Circles represent the detection probability from 25 replicates (five separate runs with five replicates per run) and the dotted line indicates the 95% limit of detection ($10^{2.91}$ $TCID_{50}$/ml blood.

Table 4 : Inter- and intra-run variation for six two-fold dilutions of EEV infected horse blood. The dashed line between −3.00 and −2.70 log10 dilution represents the limit of detection.

Samples Log$_{10}$ dilution	Results (C_T) Mean	Inter-run SD	Intra-run SD	Total SD	Total CV
3.60	34.87	2.07	0.42	1.94	0.056
3.30	35.73	0.78	0.34	0.78	0.022
3.00	36.91	2.06	0.32	1.89	0.051
2.70	37.73	1.85	0.31	1.72	0.046
2.39	38.43	0.98	0.47	0.79	0.020
2.09	39.45	0.16	0.45	0.44	0.011

C_T - cycle threshold, SD - standard deviation, CV - coefficient of variation

Both intra- and inter-run standard deviations (SD) were low with maxima at 0.47 C_T and 2.07 C_T, respectively (Table 4). The coefficient of variation (CV) ranged from 1.1 % to 5.6 %, indicating low variation between different repetitions and different runs.

4. Discussion

Equine encephalosis is a generally a mild or subclinical disease of horses. Although EEV is occasionally isolated from organs of dead horses it is not a World Organisation for Animal Health (OIE) listed disease. It does, however, play a very important role as a differential diagnosis for mild cases of AHS, a high impact disease listed by the OIE. Both diseases can present with similar clinical signs, are caused by (Orbiviruses) and are seasonal, with a peak incidence at the end of summer, when Culicoides midge numbers are at their highest. It is imperative to distinguish between AHS and equine encephalosis, as the control measures and consequences differ vastly. In South Africa, AHS is a controlled notifiable disease, whilst equine encephalosis is not.

The S7 gene of EEV is translated into the VP7 protein, which makes up the outer layer of the inner capsid of the EEV. The inner capsid is covered by the outer capsid, a layer of proteins composed of VP2 and VP5. VP7 has limited exposure on the surface of the intact virion, but is a highly immune-dominant antigen and antibodies against VP7 can be detected by an ELISA (Crafford et al., 2003).

The aim of this study was to develop a specific, sensitive, rapid and robust real-time RT-PCR assay for the detection of EEV and the S7 gene was selected as a target as it is highly conserved among serotypes and serogroups (Bremer et al., 1990; Mertens et al., 1984). As only two epitopes of the S7 gene are expressed on the surface of the virus (Anthony et al., 2007), the mutation rate of the gene due to immune pressure may be quite low.

The sequences of 38 EEV isolates, including all seven serotypes of EEV, as well as all the reference strains of EEV were obtained. The S7 gene was relatively well conserved with 92.6 - 100 % identity between the 38 isolates sequenced. The terminal ends of the S7 gene were more conserved than the middle region, similar to AHSV VP7 and NS2 sequences (Quan et al., 2010). The conserved region at the 5' end of the S7 gene (115 nucleotides) was longer than the 3' end, so the real-time RT-PCR assay was designed to target the former region.

The TaqMan® MGB™ RT-PCR assay developed for detection of EEV nucleic acid is specific and quick to perform, as it does not require visualization of the PCR products by electrophoresis. The elimination of this step saves laboratory equipment and reagents, but require a more advanced and expensive real-time thermocycler which is not available in every diagnostic laboratory. The real-time reagents are much more expensive than those used in conventional PCR, but the assay provides a convenient way of obtaining reproducible results within a short time.

The assay can be used to detect rapidly and reliably any of the EEV strains that were tested in cell or tissue culture and to distinguish EEV from AHSV. It will be useful in endemic countries where both viruses are circulating to ensure that correct control strategies are used.

As the assay was planned to be used in a multiplex format, the testing was done with a probe labeled with NED™ dye. On the StepOnePlus Real-Time PCR™ system, the efficiency of the assay appears to vary with the dye that is used. The efficiency of the same assay with 6-FAM or VIC®

appears to be much higher than with the NED™ dye (personal observation). This may explain why the efficiency of the assay was not higher and closer to 100 %.

The 95 % LOD of the assay was $10^{2.9}$ TCID$_{50}$/ml blood. There are no other published real-time RT-PCR EEV assays to compare with, but assays for AHSV have reported an end-point LOD of $10^{1.5}$ (Agüero at el., 2008), $10^{-0.90}$ (Fernández-Pinero et al., 2008) and $10^{-0.08}$ (Rodriguez-Sanchez et al., 2008) TCID$_{50}$/ml. As these are end-point LODs, the assay appears to compare favourably. Monaco et al., (2011) reported a 95 % LOD of $10^{0.33}$ TCID$_{50}$/ml for an AHSV assay, which is more sensitive than the EEV assay. It is possible that the sensitivity of the EEV could be increased with the use of different dyes, e.g. FAM or VIC® on the probes.

The results described in this study contribute to the improvement of the available techniques for the detection of EEV antigen and/or antibodies against EEV. So far, the only group-specific EEV assay is the indirect sandwich ELISA for the detection of EEV antigen (Crafford et al., 2003). The assay takes a day to obtain results. To date, there is no publishedmolecular assay for EEV and this is the first study to describe a real-time RT-PCR assay for the detection of EEV.

Acknowledgements

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.

Aharonson-Raz, K., Steinman, A., Bumbarov, V., Maan, S., Maan, N.S., Nomikou, K., Batten, D., Potgieter, C., Gottlieb, Y., Mertens, P., Klement, E., 2011. Isolation and phylogenetic grouping of equine encephalosis virus in Israel. Emerg. Infect. Dis. 17(10), 1883-1886. doi: 10.3201/eid1710.110350.

Anthony, S., Jones, H., Darpel, K.E., Elliott, H., Maan, S., Samuel, A., Mellor, P.S., Mertens, P.P., 2007. A duplex RT-PCR assay for detection of genome segment 7 (VP7 gene) from 24 BTV serotypes. J. Virol. Methods 141, 188-197.

Barnard, B.J., 1997. Antibodies against some viruses of domestic animals in southern African wild animals. Onderstepoort J. Vet. Res. 64, 95-110.

Belhouchet, M., Mohd Jaafar, F., Firth, A.E., Grimes, J.M., Mertens, P.P., Attoui, H., 2011 Detection of a fourth orbivirus non-structural protein. PLoS One 6(10):e25697. doi: 10.1371/journal.pone.0025697.

Bremer, C.W., Huismans, H., Van Dijk, A.A., 1990. Characterization and cloning of the African horsesickness virus genome. J. Gen. Virol. 71(Pt 4), 793-799.

Crafford, J.E., Guthrie, A.J., Van Vuuren, M., Mertens, P.P.C., Burroughs, J.N., Howell, P.G., Hamblin, C., 2003. A group-specific, indirect sandwich ELISA for the detection of equine encephalosis virus antigen. J. Virol. Methods 112, 129-135.

Erasmus, B.J., Adelaar, T.F., Smit, J.D., 1970. The isolation and characterization of equine encephalosis virus. Bull World Health Organ 74, 781-789.

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.

Firth, A.E., 2008. Bioinformatic analysis suggests that the Orbivirus VP6 cistron encodes an overlapping gene. Virol. J. 5:48. doi:10.1186/1743-422X-5-48.

Gorman, B.M., Taylor, J., Walker, P.J., 1983. Orbivirus, in: Joklik, W.K. (Ed.), The Reoviridae. Plenum Press, New York, pp. 287-357.

Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95-98.

Howell, P.G., Groenewald, D., Visage, C.W., Bosman, A.M., Coetzer, J.A., Guthrie, A.J., 2002.The classification of seven serotypes of equine encephalosis virus and the prevalence of homologous antibody in horses in South Africa. Onderstepoort J. Vet. Res. 69, 79-93.

Howell, P.G., Guthrie, A.J., Coetzer, J.A.W., 2004. Equine encephalosis, in: Coetzer, J.A.W., Tustin, R. (Eds.), Infectious diseases of livestock. 2nd edition. Oxford University Press, Cape Town, pp. 1247-1251.

Kalendar, R., Lee, D., Schulman, A.H., 2009. FastPCR software for PCR primer and probe design and repeat search. Genes, genomes and genomics 3(1), 1-14.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947-2948.

Mertens, P.P., Brown, F., Sangar, D.V., 1984. Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. Virology 135, 207-217.

Mildenberg, Z., Westcott, D., Bellaiche, M., Dastjerdi, A., Steinbach, F., Drew, T., 2009. Equine encephalosis in Isreal. Transbound. Emerg. Dis. 56(8), 291. doi: 10.1111/j.1865-1682.2009.01087_1.x.

Monaco, F., Polci, A., Lelli, R., Pinoni, C., Di Mattia, T., Mbulu, R.S., Scacchia, M., Savini, G., 2011. A new duplex real-time RT-PCR assay for sensitive and specific detection of African horse sickness virus. Mol. Cell. Probes 25, 87–93.

Oura, C.A., Batten, C.A., Ivens, P.A., Balcha, M., Alhassan, A., Gizaw, D., Elharrak, M., Jallow, D.B., Sahle, M., Maan, N., Mertens, P.C., Maan, S., 2012. Equine encephalosis virus: evidence for circulation beyond southern Africa. Epidemiol. Infect. 140(11), 1982-1986.

Paweska, J.T., Gerdes, G.H., Woods, P.S., Williams, R., 1999. Equine encephalosis in southern Africa: current situation, in: Wernery, U., Wade, J.F., Mumford, J.A. et al (Eds.), Equine infectious diseases. Newmarket: R & W., Newmarket, pp. 303-305.

Paweska, J.T., Venter, G.J., 2004. Vector competence of culicoides species and the seroprevalence of homologous neutralizing antibody in horses for six serotypes of equine encephalosis virus (EEV) in South Africa. Med. Vet. Entomol. 18, 398-407.

Quan, M., Lourens, C.W., MacLachlan, N.J., Gardner, I.A., Guthrie, A.J., 2010. Development and optimisation of a duplex real-time reverse transcription quantitative PCR assay targeting the VP7 and NS2 genes of African horse sickness virus. J. Virol. Methods 167, 45-52. doi: 10.1016/j.jviromet.2010.03.009.
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. doi: 10.1099/vir.0.83502-0.

Ratinier, M., Caporale, M., Golder, M., Franzoni, G., Allan, K., Nunes, S.F., Armezzani, A., Bayoumy, A., Rixon, F., Shaw, A., Palmarini, M., 2011. Identification and characterization of a novel non-structural protein of bluetongue virus. PLoS Pathog. 7(12):e1002477. doi: 10.1371/journal.ppat.1002477.

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.

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. 47 132, 115-130.

Theiler, A., 1910. Notes on a fever in horse simulating 1 horse-sickness. Transvaal Agricultural 2 J. 8(32), 581-686.

Theodoridis, A., Nevill, E.M., Els, H.J., Boshoff, S.T., 1979. Viruses isolated from *Culicoides* midges in South Africa during unsuccessful attempts to isolate bovine ephemeral fever virus. Onderstepoort J. Vet. Res. 46, 191-198.

Venter, G.J., Paweska, J.T., Williams, R., Nevill, E.M., 1999. Prevalence of antibodies against African horse sickness and equine encephalosis viruses in donkeys in southern Africa, in Wernery, U., Wade, J.F., Mumford, J.A., Kaaden, O.R. (Eds.), Proceedings 8th International Conference on Equine Infectious Diseases, 23-26 March 1998, pp 299-302.

Williams, R., du Plessis, D.H., van Wyngaardt, W., 1993. Group-reactive ELISAs for detecting antibodies to African horse sickness and equine encephalosis viruses in horses, donkey and zebra sera. J. Vet. Diagn. Invest. 5, 3-7.

Customized protocol for the MagMax^{TM} Express Particle Processor (Lifetech) for RNA extraction.

[PROTOCOL PROPERTIES]

Name = AM1830_Mod3
Protocol template version = 2.6.0
Instrument type = KingFisher
Creator = mquan
Created = 30/9/2009 9:31:36
Description = RNA isolation from whole blood.
Kit = MagMAX-96 viral RNA Isolation Kit , AM1836
Plate layouts = Default

[PLATE LAYOUTS]

Default

Plate type = KingFisher plate 200 ul
Plate change message = Change Default

A:
- volume = 20, name = Bead Mix (10 ul RNA binding beads, 10 ul Lysis/Binding Enhancer)
- volume = 50, name = Whole blood
- volume = 130, name = 65 ul Lysis/Binding Solution, 1 ul Carrier RNA, 2 ul Xeno RNA, 65 ul Isopropanol
B:
- volume = 150, name = Wash Solution 1
C:
- volume = 150, name = Wash Solution 1
D:
- volume = 150, name = Wash Solution 2
E:
- volume = 150, name = Wash Solution 2
F:
- volume = 50, name = Elution Buffer
G:
- EMPTY
H:
- EMPTY

[STEPS]

BIND
Step parameters
- Name = Lysis Binding 5 min
- Well = A, Default

Beginning of step:
- No Action = Yes

Bind parameters:

16

- Bind time = 5min 0s, speed = Fast dual mix

End of step:
- Collect beads = Yes, count = 5

WASH
Step parameters
- Name = 1st Wash I 1 min
- Well = B, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:
- Wash time = 1min 0s, speed = Fast

End of step:
- Collect beads = Yes, count = 3

WASH
Step parameters
- Name = 2nd Wash I 1 min
- Well = C, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:
- Wash time = 1min 0s, speed = Fast

End of step:
- Collect beads = Yes, count = 3

WASH
Step parameters
- Name = 1st Wash II 1 min
- Well = D, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:
- Wash time = 1min 0s, speed = Fast

End of step:
- Collect beads = Yes, count = 2

WASH
Step parameters
- Name = 2nd Wash II 1 min
- Well = E, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Wash parameters:
- Wash time = 1min 0s, speed = Fast

End of step:

- Collect beads = Yes, count = 2

DRY
Step parameters
- Name = Dry 1 min
- Well = E, Default
- Dry time = 1min 0s
- Tip position = Outside well

ELUTION
Step parameters
- Name = Elution 3 min
- Well = F, Default

Beginning of step:
- Release = Yes, time = 0s, speed = Fast

Elution parameters:
- Elution time = 3min 0s, speed = Bottom medium

Pause parameters:
- Pause for manual handling = No

Remove beads:
  - Remove beads = Yes, collect count = 5, disposal well = B

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                                30        40        50        60        70        80        90       100       110       120       130       140       150       160       170
                                ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7      GAUAGCGGCUAGAGCCCUUUCAGUUGUAAGAGCAUGUGUUACUGCACAGGAUCCACGAGCUACAAUGGCUCCUCAAGUUUUGGAAAUUUUGGGAUUAGGAAUAAACAGAUACAACGCCAUUACAGGUAGUAGAGUCACGAUGCGCCCGGG
4_2010_DVTD_E100058_01_VP7      ................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)     ...........................................................................................................A....................................
EEV5_Kyalami_VP7                ...............C..............................................................................A.......G........................G..............
EEV2_Cascara_VP7                .............................................................................................A........A...G........................G............
1_2009_DVTD_E090047_VP7 (5)     .....................................................................A.......................A........A...G.....................G..C.........
1_2009_DVTD_E090059_VP7         .....................................................................A.......................A........A...G...........U.........G..C.........
7_2008_DVTD_E080026_VP7         .....................................................................A.......................A........A...G.....................G..C.........
6_2010_DVTD_E100020_VP7         .....................................................................A...............C.A.....A.......G........U..........C..G..C.........
7_2008_DVTD_E080038_VP7 (4)     .....................................................................A.......................A........A...G.....................G..C.........
5_2009_DVTD_E090019_06_VP7 (3)  .....................................................................A.......................A........A...G.....................G..C.........
7_2008_DVTD_E080089_VP7         .....................................................................A.......................A........A...G.....................G..C.........
7_2008_DVTD_E080186_VP7 (2)     .....................................................................A.......................A........A...G.....................G..C.........
EEV4_Kaalplaas_VP7              ...........................................................................................A........A...G.....................G..C.........
2_2010_DVTD_E100043_VP7 (3)     .............................................G.......G.....................................A........A...G..................A..G..C.........
5_2009_DVTD_E090010_19_VP7      .............................................G.......G.....................................A........A...G..................A..G..C.........
EEV7_E2120_VP7                  .................C...................................G.....................................A........A...G..................A..G..C.........
EEV1_Bryanston_VP7              ...........................................................................................A........G..G.G................G..........G......
EEV3_Gamil_VP7                  ..............................................................................................C.A...G.....................G.....C..G......
EEV6_Potchefstroom_VP7          .............................................G.......G.....................................A........A...G........U..............G..C........U............
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                                180       190       200       210       220       230       240       250       260       270       280       290       300       310       320
                                ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7      GACAGUUCCAGAGAGGAAUGAUAUGAUGUUCAUGUGUAUAGAUAUGGUAUGUGCCGCGUUGAACAUUCAGUUAGGAAAUGUUUCUCCAGAUUAUACGCAGAAAUUUAGAAACGAUAGGGGUGUUGGCGACAAGUGAAAUACCCUACACUGA
4_2010_DVTD_E100058_01_VP7      ................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)     ................................................................................................................................................
EEV5_Kyalami_VP7                ..........................................................................................................................................G.....
EEV2_Cascara_VP7                ..........................................................................................U.....................................................
1_2009_DVTD_E090047_VP7 (5)     ..................................................................................................................G.........U..A.............U...
1_2009_DVTD_E090059_VP7         ..................................................................................................................G.........U..A.............U...
7_2008_DVTD_E080026_VP7         .................................................C................................................................G.........U..A................
6_2010_DVTD_E100020_VP7         ..............................................................................................C...................G.........U...................
7_2008_DVTD_E080038_VP7 (4)     .................................................C..............................................................G..A........U....G.............
5_2009_DVTD_E090019_06_VP7 (3)  .................................................C..............................................................G..A........U....G.............
7_2008_DVTD_E080089_VP7         .................................................C..............................................................G..A........U....G.............
7_2008_DVTD_E080186_VP7 (2)     .................................................C..............................................................G..A........U....G.............
EEV4_Kaalplaas_VP7              ..........................................................................................................G.......U............................
2_2010_DVTD_E100043_VP7 (3)     ......C.........................................A...C...U.....................................G.........U....G.................................
5_2009_DVTD_E090010_19_VP7      ......C.........................................A...C...U.....................................G.........U....G.................................
EEV7_E2120_VP7                  ......C.........................................A...C...U...........................G..G.................U......................................
EEV1_Bryanston_VP7              ...................................................................................................U............................................
EEV3_Gamil_VP7                  .......A....................U..............U......................................................U............................................
EEV6_Potchefstroom_VP7          ...G.....A..........................C...........C.........A.......................U..A.......C.....U...........................................
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                                330       340       350       360       370       380       390       400       410       420       430       440       450       460       470
                                ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7      UGAGGCGAUUAACGCGAUUGUGAGGGUGACAGGAGAGACGCAGACCUGGGGUCCAGUCGAUUCGCCCGAUGCCGCCGUAUCUGGGUGCGGUUGCGGUAGAAGCGCCAGGGCACUACUUUAUGCCGUUGGGAAGACAAACACAUUCAGCUUA
4_2010_DVTD_E100058_01_VP7      ................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)     ................................................................................................................................................
EEV5_Kyalami_VP7                ...........................................A................................U....................A...........G........C..
EEV2_Cascara_VP7                .......................................U..A.............U....C............U......................A...........A...........C..
1_2009_DVTD_E090047_VP7 (5)     ...........................................A...............U..........G..........A..U..U.........C.........................
1_2009_DVTD_E090059_VP7         ...........................................A...............U..........G..........A..U..U.........C.........................
7_2008_DVTD_E080026_VP7         ...........................................A...............U..........G..G.......A..U..U.........C.........................
6_2010_DVTD_E100020_VP7         ...................C.................U......A..............CU.........G..........A...U....................................C..
7_2008_DVTD_E080038_VP7 (4)     ...........................................A...............U...A......G.........A..U......A........G....C.....C..
5_2009_DVTD_E090019_06_VP7 (3)  ...........................................A...............U...A......G.........A..U......A........G....C.....C..
7_2008_DVTD_E080089_VP7         ...........................................A...............U...A......G.........A..U......A........G....C.....C..
7_2008_DVTD_E080186_VP7 (2)     ...........................................A...............U...A......G.........A..U......A........G....C.....C..
EEV4_Kaalplaas_VP7              ...........................................A...............U.........G.........A..U..U.................G........C..
2_2010_DVTD_E100043_VP7 (3)     ...........................................A...............U.........G.........A..U..................................C..
5_2009_DVTD_E090010_19_VP7      ...........................................A...............U.........G.........A..U..................................C..
EEV7_E2120_VP7                  ...........................................A...............U.........G.........A..U..................................C..
EEV1_Bryanston_VP7              .........................................G...........................C...U.....A...U..............................C..
EEV3_Gamil_VP7                  ......A....U.............................A......A.................U.............A..U..U.............G.....C.....C..
EEV6_Potchefstroom_VP7          ........C..U........A...............A....A......U........A..................U.........A..U..U..C........A...................C..
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                                     480       490       500       510       520       530       540       550       560       570       580       590       600       610       620
                                     ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7           CACUGAUUCCAACACUAUUCAGGUGAGCCUUACGCCGAACAGUGCUGCGCAAGUCAACGGCGCUAUGACGCCGCAAGGUGUGGGAGCUCGUGCAGUUAUUUUUUGUAUGGAGACCUUUUGCAAUGUACACAAGUCAGGCGGGAGCAGCCGU
4_2010_DVTD_E100058_01_VP7           ..............................................................................................A.......................................................
4_2008_DVTD_E080129_VP7 (7)          ....................................................................................................................................................
EEV5_Kyalami_VP7                     ...........................C..................................................................G.........A.............................................
EEV2_Cascara_VP7                     ......C.....................C..........................A......................................G.......U.A.........................................U..
1_2009_DVTD_E090047_VP7 (5)          U.............................U...........A.........U.........................................G...........A.......................A..................U..
1_2009_DVTD_E090059_VP7              U.............................U...........A.........U.........................................G...........A.......................A..................U..
7_2008_DVTD_E080026_VP7              U.......G.....................U...........A...........C.......................................G...........A.......................A..................U..
6_2010_DVTD_E100020_VP7              ..............................U...........A..................................................G...........A.......................A..................U..
7_2008_DVTD_E080038_VP7 (4)          U.........................................A..................................................G...........A.........U...........................G......U..
5_2009_DVTD_E090019_06_VP7 (3)       U.........................................A..................................................G...........A.........U...........................G......U..
7_2008_DVTD_E080089_VP7              U.........................................A..................................................G...........A.........U...........................G......U..
7_2008_DVTD_E080186_VP7 (2)          U.........................................A..................................................G...........A.........U...........................G......U..
EEV4_Kaalplaas_VP7                   U.........................................................................................................G.......................................U..
2_2010_DVTD_E100043_VP7 (3)          ........G....C............................A......G......................................G...........A.............................................
5_2009_DVTD_E090010_19_VP7           ........G....C............................A......G......................................G...........A.............................................
EEV7_E2120_VP7                       U............A...........................A......G......................................G...........A.............................................
EEV1_Bryanston_VP7                   U..C.................C....................A..................................................G.......A....A....C..................................G...
EEV3_Gamil_VP7                       U.....C.............C....A....C..........A....G.......................................G.......A....A.............................................
EEV6_Potchefstroom_VP7               U..............C....A....................A..................................................G...A..U..A..A......................G........C......G.......

                                     630       640       650       660       670       680       690       700       710       720       730       740       750       760       770
                                     ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7           AGCUCAACCUCCCGGCGUGACUUUGUCGUUGGGUGGGAGUUAAUAUGCCAGAGGGUAGAAUUCUGAUAUGGGACGGUGUUGCUCCAGUUGUUGUACAAAAUCCAGGGAACGCGCCAGCUAUGGCUCAAAUUGAAGUCGUGAGAUUCACACA
4_2010_DVTD_E100058_01_VP7           ....................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)          ....................................................................................................................................................
EEV5_Kyalami_VP7                     ....................................................................................................................................................
EEV2_Cascara_VP7                     ....................................A........................................................U......................................................
1_2009_DVTD_E090047_VP7 (5)          .......A..........G....................C........................................................................................................
1_2009_DVTD_E090059_VP7              .......A..........G....................C........................................................................................................
7_2008_DVTD_E080026_VP7              ..................A....................C.................................................................................................G.......
6_2010_DVTD_E100020_VP7              ................A..A...................C.......................................................................................G.....G.
7_2008_DVTD_E080038_VP7 (4)          .....G..C.........A....................C.................................................................................................G.......
5_2009_DVTD_E090019_06_VP7 (3)       .....G..C.........A....................C.................................................................................................G.......
7_2008_DVTD_E080089_VP7              .....G..C.........A....................C.................................................................................................G.......
7_2008_DVTD_E080186_VP7 (2)          .....G..C.........A....................C.................................................................................................G.......
EEV4_Kaalplaas_VP7                   ..................A....................C.................................................................................................G.......
2_2010_DVTD_E100043_VP7 (3)          ..................A....................C...................................................C..........................................
5_2009_DVTD_E090010_19_VP7           ..................A....................C...................................................C..........U.............................
EEV7_E2120_VP7                       ..................A....................C........................................................................................................
EEV1_Bryanston_VP7                   ......G...........G........................................U.........................................................................U..A.....U.....
EEV3_Gamil_VP7                       ......G......U....A.......................................................C......G....C....C...................................................A......
EEV6_Potchefstroom_VP7               ..................A..A.....A...............................G..C..................C..........G.....G....U.............................U..A.......

                                     780       790       800       810       820       830       840       850       860       870       880       890       900       910       920
                                     ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7           GCUCCUACACUCAUAUGAGACGGUUCCAGAAACCCGUGAGAAUAUGGCUAGAUGCUACUCAUUCCUUAGUGUUACGUGGCACACCCUCCGAUCAGCUAUUCUGAGGUCCUUAGGAAUGCUUCCAGUGCACCAGUCCGCCUAUCCACCGAC
4_2010_DVTD_E100058_01_VP7           ..................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)          ..................................................................................................................................................
EEV5_Kyalami_VP7                     ...........................................................................U.....................A......................A.....U..C......
EEV2_Cascara_VP7                     .................G.........................................................U.....................A......................U..C......
1_2009_DVTD_E090047_VP7 (5)          ..................U.....G..................................................U.....................A....G.................U..C.....A.
1_2009_DVTD_E090059_VP7              ..................U.....G..................................................U.....................A...C.G.................U..C.....A.
7_2008_DVTD_E080026_VP7              ........................G..................................................U.....................A......G..............U..C.....A.
6_2010_DVTD_E100020_VP7              ..............C.........G..................................................U.....................A......G..G............U..C.....A.
7_2008_DVTD_E080038_VP7 (4)          ........................G..................................................U...C.....A...G.......U..U..C......
5_2009_DVTD_E090019_06_VP7 (3)       ........................G.......................................A.........U...C.....A...G.......U..U..C......
7_2008_DVTD_E080089_VP7              ........................G..................................................U...C.....A...G.......U..U..C......
7_2008_DVTD_E080186_VP7 (2)          ........................G..................................................U...C.....A...G.......U..U..C......
EEV4_Kaalplaas_VP7                   ........................G..................................................U...C.....A...G.......U..U..C......
2_2010_DVTD_E100043_VP7 (3)          ............G.......A....G.....C..A...............................U.............A..U..G.......U..C......
5_2009_DVTD_E090010_19_VP7           ............G.......A....G.....C..A...............................U.............A..U..G.......U..C......
EEV7_E2120_VP7                       ............G.......A....G.....C..A...............................U.............A..U..G.......U..C......
EEV1_Bryanston_VP7                   ..........................U...............................U.............A..C.........C.......U..C......
EEV3_Gamil_VP7                       ..........G.............U...........U..................U.............A..C.......U..C......
EEV6_Potchefstroom_VP7               ............C....A.......G.....A.................C.................G........A..C...........A..........U..C......
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                                    930       940       950       960       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070
                                    ..|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
4_2009_DVTD_E080010_05_VP7          GAAUAGGUAUGAAUAUCUCGCUUACCUUUUAAUUGCAGCACUAGCGGAUGCAUACGACGCUUUGAGACCAAACUUUGAUAUGUUUAAUCAGCCAAAUGUGCCUCUGCAGCCAACAAGGGCUCAAAUCGCGGCGUUGUACCGCUAAACCGA
4_2010_DVTD_E100058_01_VP7          ................................................................................................................................................
4_2008_DVTD_E080129_VP7 (7)         ................................................................................................................................................
EEV5_Kyalami_VP7                    ...................C...........C................................................................................................................
EEV2_Cascara_VP7                    ...................C.....C......................................................................................................................
1_2009_DVTD_E090047_VP7 (5)         ...................C.....C.....................................G..U...............................................................................
1_2009_DVTD_E090059_VP7             ...................C.....C.....................................G..U...............................................................................
7_2008_DVTD_E080026_VP7             ...................C.....C.......G.............................G..U...............................................................................
6_2010_DVTD_E100020_VP7             ...C...............C............G.............................G..U...............................................................................
7_2008_DVTD_E080038_VP7 (4)         ...................C.....C.....................................G.............................G...................................................
5_2009_DVTD_E090019_06_VP7 (3)      ...................C.....C.....................................G.............................G...................................................
7_2008_DVTD_E080089_VP7             ...................C.....C.....................................G.......................A..G......................................................
7_2008_DVTD_E080186_VP7 (2)         ...................C.....C.....................................G.............................G...................................................
EEV4_Kaalplaas_VP7                  ...................C.....C.....................................G.................................................................................
2_2010_DVTD_E100043_VP7 (3)         ...C...............C.....C.....A....G.........................G........A.........................................A.............................A.
5_2009_DVTD_E090010_19_VP7          ...C...............C.....C.....A....G.........................G........A.........................................A.............................A.
EEV7_E2120_VP7                      ...C...............C.....C.....A....G.........................G........A.........................................A.............................A.
EEV1_Bryanston_VP7                  ...................C.....C.....A................................................................................................................
EEV3_Gamil_VP7                      A.GC...............C.............G....A...................................A..............A.............................G.........................
EEV6_Potchefstroom_VP7              ...........U......C......G.......G..G..A.....G.......C..A.......U.......................................................A.........................
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                                    1080      1090      1100      1110      1120      1130      1040
                                    ..|....|....|....|....|....|....|....|....|....|....|....|....|.....
4_2009_DVTD_E080010_05_VP7          CAUGUUUUCCACAGUACAUGUAGGAGGCGGAGUACAUGCGCAUCGGUUGGUAUACUAACAGUCUGUAUAUUUU
4_2010_DVTD_E100058_01_VP7          .......................................................................
4_2008_DVTD_E080129_VP7 (7)         .......................................................................
EEV5_Kyalami_VP7                    .......................................................................
EEV2_Cascara_VP7                    ..............G........................................................
1_2009_DVTD_E090047_VP7 (5)         .......................................A.................G....
1_2009_DVTD_E090059_VP7             .......................................A.................G....
7_2008_DVTD_E080026_VP7             .......................................A.......................
6_2010_DVTD_E100020_VP7             .......................................A.......................
7_2008_DVTD_E080038_VP7 (4)         .......................................A.......................
5_2009_DVTD_E090019_06_VP7 (3)      .......................................A.......................
7_2008_DVTD_E080089_VP7             .......................................A.......................
7_2008_DVTD_E080186_VP7 (2)         .......................................A.......................
EEV4_Kaalplaas_VP7                  .......................................A.......................
2_2010_DVTD_E100043_VP7 (3)         .............G.........................A.......................
5_2009_DVTD_E090010_19_VP7          .............G.........................AA......................
EEV7_E2120_VP7                      .............G.........................A.......G...............
EEV1_Bryanston_VP7                  .............G.........................A.......G...............
EEV3_Gamil_VP7                      .............G.................................................
EEV6_Potchefstroom_VP7              .......................................................................
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Figure S1. EEV S7 (VP7) gene sequences. Dots indicate identity and letters differences with the first sequence in the group. Identical sequences are represented only once in the figure and the number of identical sequences are indicated in brackets after the name of a random sequence in the group. The location of the real-time RT-PCR assay primers and probe are indicated by grey shaded boxes.