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

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A B S T R A C T

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 neutralisation 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: $10^{2.7}$ to $10^{3.3}$).

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

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 85% in donkeys (Venter et al., 1999). In Thoroughbred horses the seroprevalence of neutralising 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 (Williams et al., 1993; Barnard, 1997).

EEV infection in horses was been reported recently in Israel, and involved approximately 150 cases with no reported mortalities (Mildenberg et al., 2009; Aharonson-Raz et al., 2011). 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 haemorrhagic 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

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a viral protein, namely seven structural proteins (VP1 to VP7) and non-structural proteins (NS1, NS2, NS3/NS3a, NS4) (Mertens et al., 1984; Firth, 2008; Belhouchet et al., 2011; 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 neutralisation 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 recognised 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 5417 C 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 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 visualised 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 H_2O. 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 labelled 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 (%) = $100 \times (10^{1/\text{slope}} - 1)$.

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.