Quantitative RT-PCR assays for identification and typing of the Equine encephalosis virus

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Abstract

Equine encephalosis (EE) is an acute, arthropod-borne, noncontagious, febrile disease of equids. The clinical signs of EE are similar to milder forms of African horse sickness (AHS) and the two diseases can be easily confused. The Equine encephalosis virus (EEV) is a distinct virus species within the genus *Orbivirus*, family *Reoviridae*, with ten linear segments of dsRNA genome. Seven distinct serotypes of EEV have been recognised on the basis of sequence analyses of Seg-2. The need for differential diagnosis of similar forms of EE and AHS warranted the development of molecular diagnostic methods for specific detection and identification of EEV. We report the development of quantitative real-time RT-PCR assay for detection of any member of the EEV species targeting the highly conserved EEV Seg-9. Similar serotype-specific qRT-PCR assays were designed for each of the seven EEV serotypes targeting genome Seg-2, encoding the serotype determining VP2 protein. These assays were evaluated using different EEV serotypes and other closely related orbiviruses. They were shown to be EEV virus species-specific, or EEV type-specific capable of detecting 1 to 13 copies of viral RNA in clinical samples. The assays failed to detect RNA from closely related orbiviruses, including AHSV and Peruvian horse sickness virus (PHSV) isolates.

Keywords Equine encephalosis · Equine encephalosis virus · EEV · TaqMan assays · Real-time RT-PCR · Serotype-specific assays · Serogroup-specific assays · Orbivirus Reference Collection · *Culicoides*

Introduction

Equine encephalosis (EE) is a noncontagious acute febrile disease of equines caused by the Equine encephalosis virus (EEV), a member of the genus *Orbivirus*, family *Reoviridae*.

Like many other orbiviruses, EEV is transmitted between its equine hosts by the bites of infected *Culicoides* midges [1]. The clinical signs of EE are usually mild but may include mild fever, inappetence and listlessness to high temperature, oedema of the face, congested mucosa, respiratory distress and

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cardiac signs. Early abortion during the first 5–6 months of gestation due to EEV infection can potentially lead to a misdiagnosis as infertility in a mare [2]. The clinical signs of EEV infection are similar to those of other notifiable equine diseases and can be confused with milder forms of African horse sickness (AHS) which is usually fatal [3, 4].

EEV was first isolated in 1967 from a thoroughbred mare named Cascara from the Kimberley district of South Africa [5]. EEV was initially thought to be endemic in and restricted to southern Africa with sero-epidemiological evidence for EEV-specific antibodies in 77 to 84% of horses in South Africa [2, 6] with a high incidence of EEV infection in thoroughbred foals between the age of 4 and 7 months after they had lost their maternal immunity [7]. After 1967, seasonal outbreaks of EEV took on epidemic proportions in southern Africa [8] raising concerns about the risk of its spread to other countries where susceptible hosts and competent vectors are present. Subsequent serological studies indicated that like AHSV, EEV infection is now widespread in East and West African countries [4]. In July 2008, the National Institute of Virology, India, isolated and identified EEV-4 (Bryanston) in necropsy samples from a horse in Pune. In 2008, EE outbreaks were reported in Israel, Palestine and Jordan [8, 9] along with serological evidence that it has been circulating in the Middle East since 2001 [4]. A recent study has also indicated the potential for further emergence of EEV into new ecologic niches in Europe [10].

The long-distance movement of viruses (including different orbiviruses) has been identified between continents and has been linked to increased human travel and international trade [11–13]. Several distinct and novel orbiviruses have been detected or isolated in South America, although the threats that they pose to the health of domesticated and wild animals (including horses) have not been fully evaluated [14]. Peruvian horse sickness virus is a mosquito-transmitted orbivirus first isolated in 1979 (with a case fatality rate of ~78%) that shows a high level of nucleotide sequence similarity to the Elsey Station virus (ELSV) from Australia [15]. The Rioja virus, which was also isolated from donkeys and mosquitoes in Peru, is closely related to Yunnan orbivirus from China [14], indicating that simple geographic separation is not sufficient to completely remove the risk of *Orbivirus* introductions and emergence of the diseases they cause [15, 16]. Although Brazil currently appears to be free of EEV, it has large arthropod populations of known *Orbivirus* vector species as well as susceptible equine hosts and any future introductions could cause significant disease outbreaks.

Like other orbiviruses, the EEV genome contains 10 segments of linear double-stranded (ds) RNA (Seg-1 to Seg-10) which collectively encode 7 structural proteins (VP1 to VP7) and 5 non-structural proteins (NS1 to NS5) [17, 18]. The orbivirus Seg-10 encodes two related proteins (NS3 and NS3a) in the same open reading frame (ORF) as well as

NS5 in a second downstream ORF [19]. Seg-9 also encodes two different proteins (VP6 and NS4) in different ORFs [17, 20]. However, the other genome segments are currently thought to be monocistronic.

There are seven distinct serotypes of EEV that can be distinguished in serum neutralisation tests [2]. Another method of serotyping is plaque inhibition neutralisation assay [21]. Like the bluetongue virus (BTV), the *Orbivirus*-type species, the larger EEV outer-capsid and cell attachment protein VP2, is encoded by Seg-2. Consequently, Seg-2 and VP2 show sequence variations that correlate with and can be used to identify the virus-serotype [22]. In contrast, the viral helicase VP6 (encoded by Seg-9), is much more conserved within each orbivirus species [23]. The ability of orbiviruses within a single virus species to exchange genome segments by reassortment has resulted in an absence of any detectable correlation between sequence variation in Seg-9 (VP6) and the virus serotype.

Historically, EEV was identified by virus isolation (in BHK cells, suckling mice brain or embryonated chicken eggs) [5] and by serological methods [21]. EEV virus group-specific antibodies can also be detected by cELISA [24], while virus neutralisation test (VNT) [6] is currently being used for EEV serotype determination.

Molecular assays which are extremely specific and sensitive are increasingly being used as primary diagnostic tools for identification of other *Orbivirus* serogroup and serotypes as well as epidemiological investigations [25–30]. These methods, which can detect and identify the dsRNA viral genome by conventional gel-based reverse transcription polymerase chain reactions (RT-PCR) [27]; real-time RT-PCR (qRT-PCR) assays [29, 31]; reverse transcription loop-mediated isothermal amplification (RT-LAMP) [32]; sequence analyses and phylogenetic comparisons [33, 34] are more sensitive and more specific than serological diagnostic techniques and can provide an accurate quantitative assessment of the relatedness of different viruses. Although a qRT-PCR has recently been developed for detection of EEV genome Seg-7 [35], no qRT-PCR assays are as yet available for the identification of EEV serotype.

An ability to rapidly and accurately detect, identify and distinguish viruses responsible for disease outbreaks in livestock (including horses) is essential for the design and rapid implementation of appropriate control strategies. This paper describes the development and evaluation of Seg-9-based virus species/serogroup and Seg-2-based serotype-specific qRT-PCR assays for accurate detection and identification of EEV virus isolates. The virus species-specific assay, which detects all seven serotypes of EEV and can distinguish them from AHSV or PHSV strains was designed based on Seg-9. A set of seven individual ‘type-specific’ assays were also designed to detect and identify Seg-2 of each EEV serotype. Assay specificity was evaluated using EEV and other *Orbivirus* isolates available from the Orbivirus Reference Collection (ORC), located at The Pirbright Institute.

Material and methods

Primers and probe design for qRT-PCR assays

The sequences of Seg-2 and Seg-9 of EEV strains that are available in GenBank (Table 1) were aligned using Clustal X2 [36] or MEGA 5 [37]. Primers and probes for a virus species-specific qRT-PCR assay were designed according to TaqMan specifications, targeting a conserved region identified in Seg-9 of all isolate/serotypes (Table 2). In contrast, primers and probes for Seg-2-based serotype-specific qRT-PCR assays were designed targeting a highly variable region of Seg-2 was identified in each of the different serotypes (Table 2). The probes were labelled at their 5' and 3' ends with FAM and Black Hole Quencher-1 (BHQ-1) respectively. All oligonucleotides were synthesised by Eurogentec, UK. The sequences of the different primers and probes were evaluated in silico with cognate genome segments of all 28 *Orbivirus* species to ensure no cross-reactions with closely related viruses [38] (Table 1—Supplementary data).

Virus isolates

Thirty-one isolates, including six reference strains of EEV, and several field strains from Israel and Gambia were used for an initial validation of the qRT-PCR assays (listed at http://www.reoviridae.org/dsRNA_virus_proteins/ReoID/EEV-isolates.htm) [39]. The samples used to generate isolates were taken from naturally infected animals in the field, by qualified veterinarians, as part of normal veterinary care and diagnostic testing procedures in the respective countries. Representative isolates of other orbiviruses were also used to assess assay specificity. All of the isolates used in the study were obtained from the ORC at The Pirbright Institute (http://www.reoviridae.org/dsRNA_virus_proteins/reoid/EEV-isolates.htm) [39] and were propagated in BHK-21 clone 13 cells (European Collection of Animal Cell Cultures [ECACC – 84100501]), Vero cell monolayers (ECACC – 84113001) or

in KC cells derived from *Culicoides sonorensis* [40]. Infected mammalian cells were harvested when 100% cytopathic effect (CPE) was observed, while KC cells were harvested 7 days post infection.

The Seg-9-based serogroup-specific and Seg-2-based serotype-specific qRT-PCR assays could not be validated using an isolate of EEV serotype 7 (Northrand) as this was not available within our laboratory, or from the ORC. The serogroup assay was therefore evaluated in silico while the Seg-2-based serotype-specific assay was tested using a PCR amplicon (105 bp) generated from recombinant plasmid containing EEV-7 (Northrand/E21/20) Seg-2 in pGEMT vector (pGEMT-EEV7-VP2). The PCR was performed using KOD polymerase (Novagen) and the primers which were used for qRT-PCR (Table 2).

RNA sample preparation

Viral genomic dsRNA was extracted from EEV infected cells using the Trizol Reagent® (Invitrogen, UK) [41], or from tissue culture supernatant, blood and other clinical samples, using a MagVet™ universal purification kit (Kingfisher™, Life Technologies). EEV-7 Seg-2 DNA (105 bp) generated from PCR was purified using illustra GFX PCR DNA and gel band purification kit (GE Healthcare) and quantified using NanoDrop (Thermo Fisher Scientific, USA).

qRT-PCR reactions

The qRT-PCR assays used the SuperScript III/ Platinum Taq One-Step qRT-PCR Kit (Invitrogen, UK). The reaction mix contains 1 µM of each of primer and ROX, 0.1 µM of hydrolysis probe, 5 mM of MgSO₄ and 1 unit of Taq polymerase. dsRNA samples were heat denatured prior to addition to the qRT-PCR reaction mix. In order to achieve low cycle threshold (Ct) values and high fluorescence signals, the individual steps of the method were optimised for each assay including primer concentrations, primer-probe ratios, magnesium

Table 1 Sequence data used to design EEV qRT-PCR assays

Virus serotype	Name	Year of first isolation	ORC reference number ²	Seg-9 acc. number	Seg-2 acc. number
Reference strains [1]					
EEV-1	Cascara	1967	RSA1967/03	HQ630917	HQ630913
EEV-2	Gamil	1971	RSA1971/06	HQ630927	HQ630923
EEV-3	Kaalplaas	1974	RSA1974/06	HQ630937	HQ630933
EEV-4	Bryanston	1976	RSA1976/03	HQ630907	HQ630903
EEV-5	Kyalami	1976*	RSA1976/06	HQ630947	HQ630943
EEV-6	Potchefstroom	1991	RSA1991/03	HQ630897	HQ630893
EEV-7	Northrand/ (E21/20)	2000	–	HQ630957	HQ630953

* Isolated in 1974, but positively identified as new serotypes in 1976

Table 2 List of primers and probes for pan-EEV and EEV type-specific assays

Assay type and serotype	Oligo name	Oligo sequence (5'-3')
Seg-9 based / Pan-EEV assay		
EEV-1-EEV-7	EEV/S9/392-407F	GGATCAAACGGTGGAG
	EEV/S9/493-475R	AACTTCCTTTCTCCTTCA
	EEV/S9/440-416P	CCTTCTCTTCACCCCTTGGCCCTCC
Seg-2 based/type specific		
EEV-1	EEVcas/S2/389-362P	CTTATCCCTGGTTACTTTTGCTTCTTTT
	EEVcas/S2/302-317F	GCGGCTTGATAGGGAA
	EEVcas/S2/413-397R	TACCATTCCAAAACGAG
EEV-2	EEVgam/S2/403-432P	CCTGGATAGTACGAGGCCACTACACA GAAC
	EEVgam/S2/375-391F	GAGATCCGACGTAAGCA
	EEVgam/S2/473-452R	TGCGTTACACATTAAAGCGAAT
EEV-3	EEVkaa/S2/399-373P	CCTGCTTCTCTCCACTAGTTGCTTCC
	EEVkaa/S2/352-369F	GTAAACTGACAAAGGCTG
	EEVkaa/S2/431-412R	GCCATTAACCTTCATGAATG
EEV-4	EEVbry/S2/195-222P	CATACCCAACGACAATACATTCGCAGGC
	EEVbry/S2/156-174F	GCTAGATCAGCAAGAAGAT
	EEVbry/S2/246-226R	TAGATTCTTAGGCCACGCGTT
EEV-5	EEVkya/S2/398-372P	CCTAAAAGATTCCCCTCCCTCAATCGC
	EEVkya/S2/333-352F	ATTGGTTTCCATTAAATACG
	EEVkya/S2/416-400R	AACCGGGTCTTCTCCAG
EEV-6	EEVpot/S2/407-382P	CCCATTAACCTCTTTGAGCCTTGCCC
	EEVpot/S2/338-356F	TTGTGTCGTAAAGTATGG
	EEVpot/S2/433-419R	GCTCATCCCTTCTGG
EEV-7	EEVnor/S2/394-368P	CCTCTGTTCACGCTTCGATAGTTCC
	EEVnor/S2/325-341F	GTTCAACACAGCTGGCT
	EEVnor/S2/430-413R	TATCGCGATGCCTCAACT

The names of the oligonucleotide primers and probes indicate the group specificity or type specificity of the assay. S9 indicate the group-specific assays while S2 indicate type-specific assays. FP, RP or P stand for 'forward primer', 'reverse primer' and 'probe' respectively. The numbers represent annealing positions on the genome segment. All probes are with 5'FAM and 3'BHQ modifications

concentration, reverse transcription temperature, reverse transcription period, annealing temperature, and annealing period. The composition of the individual optimised assays is presented in Table 3. To avoid accidental contamination, assembly of the reaction mix was conducted in a laminar flow hood dedicated to 'clean work', separate to an RT-PCR assembly hood. Amplification was carried out for all assays in an MX3005p PCR machine (Stratagene, UK) using the following conditions: 55 °C for 30 min, 95 °C for 10 min followed by 50 cycles of 95 °C for 30 s and 60 °C for 1 min. Fluorescence was measured during the 60 °C annealing/extension step. Cycle threshold (Ct) values were measured at the point at which the sample fluorescence signal crossed a threshold value (the background level). Negative results (for assays that did not exceed this level of signal) are reported as 'No Ct'. The Ct should be located in the exponential phase of amplification. If the threshold is located too close to the baseline, nonspecific spikes in fluorescence and aberrant fluorescent traces will be

Table 3 RT-PCR assay composition

Group-specific assay	Amount (μl)
Forward primer (25 μM)**	1.0
Reverse primer (20 μM)	1.0
Probe (5 μM)	0.5
ROX(1/10 dilution)	0.5
50 mM MgSO ₄ *	1.0
SuperScript III RT/Platinum <i>Taq</i> Mix*	0.5
2× reaction mix*	12.5
Nuclease-free water	3.0
dsRNA	5.0
Total volume (μl)	25

*SuperScript III/ Platinum *Taq* One-Step qRT-PCR Kit (Invitrogen, UK)

**Forward primer (20 μM) was used for type-specific assays

interpreted as positive. If the threshold is located above the exponential phase (in the linear phase), weak positive specimens with a low level of amplification will be interpreted as negative and no Ct value will be reported. The RNA positive control was diluted to a working concentration that will consistently give a target Ct of approximately 25.0. Any test run where the positive control has a Ct higher than 29.0 was repeated to assure that test reproducibility is maintained.

Diagnostic specificity

Virus isolates belong to EEV serotypes 1 to 6 (Table 4) were tested in triplicates, using the Seg-9 virus species-specific assay. Representative isolates of other *Orbivirus* species,

including BTV, AHSV, Epizootic haemorrhagic disease virus (EHDV) and Peruvian horse sickness virus (PHSV) (Table 4) were also tested to assess its diagnostic specificity. Total nucleic acid extracted from uninfected BHK or KC cell culture supernatants, horse blood, or homogenised adult *C. sonorensis* were also tested to ensure that there were no false positives caused by cross-reactions with the host-species, with uniformly negative results. The Seg-2-based assays (apart from the EEV-7 assay) were all tested with RNA extracted from the homologous serotype, as well as with heterologous serotypes, to ensure their specificity and diagnostic sensitivity. Although an isolate of EEV-7 was not available, Seg-9 of EEV is not thought to influence serotype. The ability of the Seg-9 group-specific assay to detect this serotype was

Table 4 Specificity of EEV group- (Seg-9) and type-specific (Seg-2) qRT-PCR assays

EEV serotype [ORC isolate number]	Group-specific	EEV type-specific assays (Seg-2)						
	Seg-9	EEV-1	EEV-2	EEV-3	EEV-4	EEV-5	EEV-6	EEV-7
EEV-1 [RSA1967/03]	14.4	11.9	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
EEV-2 [RSA1971/06]	20.43	No Ct	13.54	No Ct	No Ct	No Ct	No Ct	No Ct
EEV-3 [RSA1974/06]	13.2	No Ct Ct	No Ct	11.59	No Ct	No Ct	No Ct	No Ct
EEV-4 [RSA1976/03]	15.41	No Ct	No Ct	No Ct	12.49	No Ct	No Ct	No Ct
EEV-5 [RSA1976/06]	16.68	No Ct	No Ct	No Ct	No Ct	12.97	No Ct	No Ct
EEV-6 [RSA1991/03]	14.21	No Ct	No Ct	No Ct	No Ct	No Ct	12.42	No Ct
EEV-7 (E20/21)	–	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	18.11
Field strains								
GAM2009/06	24.05	No Ct	No Ct	27.44	No Ct	No Ct	No Ct	No Ct
GAM2009/05	24.87	No Ct	No Ct	26.14	No Ct	No Ct	No Ct	No Ct
A113/10-01 (ISR2009/20)	18.46	No Ct	No Ct	17.26	No Ct	No Ct	No Ct	No Ct
A113/10-02 (ISR2009/20)	16.97	No Ct	No Ct	17.65	No Ct	No Ct	No Ct	No Ct
A113/10-03 (ISR2009/21)	19.01	No Ct	No Ct	17.53	No Ct	No Ct	No Ct	No Ct
A113/10-04 (ISR2009/21)	14.68	No Ct	No Ct	17.59	No Ct	No Ct	No Ct	No Ct
Other orbiviruses								
BTV-1-24 reference strains	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
AHSV 1-9 reference strains	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
EHDV 1-9 reference strains	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
Peruvian horse sickness virus (PHSV) (PER1997/01)	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
Chobar Gorge virus (CGV) (NEP1970/01)	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
Umatilla virus (UMAV) (USA1969/01)	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
Tracambe virus (TRV) (BRA1985/01)	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
Palyam virus (PALV) (SUD1983/09)	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
Uninfected controls								
Uninfected Horse blood	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
Uninfected BHK cells	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
Uninfected Vero cells	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct
Uninfected KC cells	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct	No Ct

ND not done

BTV 1–24, EHDV 1–9 (including 3 and 8), AHSV 1–9; no Ct in all these reactions

therefore evaluated *in silico* based on the published Seg-9 sequence of an original isolate of this serotype (accession number HQ630957).

Assay sensitivity and efficiency

The analytical sensitivity of the assays was assessed using a dilution series of quantified genomic dsRNA of the EEV isolates as previously described for BTV, AHSV and EHDV [31, 42]. The dsRNA standards were prepared as follows: viral dsRNA of the six available serotypes of EEV was extracted using Trizol as previously described [41], ssRNA was digested with RNase I_f (New England Biolabs), purified using a phenol-chloroform method, assessed for ‘quality’ by 1% agarose gel electrophoresis and the concentration of dsRNA was determined using a NanoDrop (Thermo Fisher Scientific, USA). As an EEV-7 virus isolate was not available, the specificity and sensitivity of the EEV-7 type-specific assay was assessed using a PCR amplicon (105 bp) generated from a recombinant plasmid DNA (pGEMT-EEV7-VP2) containing Seg-2 of EEV-7 (Northrand/E21/20).

The number of dsRNA copies was calculated with the formula: $Y = (X/(N \times 680)) \times 6.022 \times 10^{23}$, where Y = molecules/ μ l; X = g/ μ l dsRNA; N = viral genome length in nucleotides; 680 is the average molecular weight per nucleotide base pair of dsRNA. Serial 10-fold dilutions of viral dsRNA of EEV-1 to EEV-6 and that of the EEV-7 cDNA containing plasmid (10^9 to 10^0 copies per μ l) were made in nuclease-free water containing carrier RNA, then tested in triplicates. However, for the Seg-9-based group-specific assay, the serial 10-fold dilutions of viral dsRNA of EEV-4 (Bryanston) (10^7 to 10^0 copies per μ l) were made in nuclease-free water containing carrier RNA, then tested in triplicates. The efficiency of the qRT-PCR assay was estimated on the basis of standard curve plotting Ct values against corresponding log dsRNA copy number per reaction. PCR efficiency was determined by the formula: PCR efficiency $E\%$ = $(10^{-1/\text{slope}} - 1) \times 100$.

To confirm the reproducibility of assays, intra-assay (each sample tested three times within an experiment) and inter-assay (each sample tested one time in three different experiments) variations were evaluated.

Results

Sequence comparison and assay design

Publicly available and newly generated nucleotide sequences of Seg-2 and Seg-9 from these reference strains of the different EEV serotypes were compared (Table 1). A conserved region was identified in Seg-9, providing target footprints for primers and probe for an EEV species-specific RT-PCR assay. Unique regions and target footprints for primers and probes that are

specific for each EEV serotype were identified within the Seg-2 sequences, for the development of type-specific qRT-PCR assays (Table 2). *In silico* comparisons with other orbiviruses sequences did not show any similarity in the footprints for these primers and probes indicating that they are specific for EEV.

Evaluation of assay specificity

A panel of six monotypic reference strains, representing EEV-1 to EEV-6 (Table 1) were tested in triplicates with the EEV species-specific qRT-PCR assay targeting Seg-9 and ‘typing assays’ targeting Seg-2 (Table 4). All six serotypes were detected in species-specific qRT-PCR and in type-specific assays amplification was only observed with the homologous assay (Table 4). The specificity of these assays was further evaluated using different field samples and isolates of EEV collected from different geographical locations. The group assay detected all EEV tested and type-specific assays produced positive signals only with homologous serotypes (Table 4), while multiple reference serotypes of BTV, AHSV, EHDV and other orbiviruses all gave negative results (Table 4). ‘No Ct’ results were also consistently generated with nucleic acid samples from *C. sonorensis*, uninfected horse bloods or cell culture supernatants (KC cells, BHK and Vero cells) (Table 4). Due to the unavailability of an EEV-7 isolate, the group-specific assay was not directly evaluated for this serotype. However, based on the bluetongue virus model, Seg-9 of EEV does not directly influence serotype and an *in silico*-based analysis indicate that the assay would have detected Seg-9 from an original isolate of this type (accession number HQ630957). The Seg-9 qRT-PCR assay is capable of detecting all seven EEV serotypes but did not detect any of the isolates tested from other *Orbivirus* species. It is therefore considered to be EEV-specific. The EEV-7 type-specific assay gave a positive signal only with the DNA copy derived from recombinant plasmid (pGEMT-EEV7-VP2) containing EEV-7 VP2 of the homologous serotype. The specificity of this EEV-7 assay was also evaluated and confirmed *in silico* based on the Seg-2 accession numbers (Table 1). These RT-PCR results were confirmed by sequencing and phylogenetic analyses of Seg-2 for the majority of the field isolates tested as listed in Table 4.

Assay sensitivity and efficiency

Efficiency rates for the Seg-9 assay were calculated based on a dilution series of viral RNA purified from EEV-4 (Bryanston) showing values of 91.3% efficiency estimated over nine (10^1 – 10^9) log dilutions. Over these dilution ranges, a linear correlation (R^2) of 0.999 was observed (Table 5 and Fig. 1). The

Table 5 Analytical sensitivity and efficiency of EEV group–(Seg-9) and type–specific (Seg-2) qRT-PCR assays

Assay	R^2	Efficiency (%)	Ct	Slope	Detection limit (copy number)
EEV group–specific assay (seg-9)	0.999	91.3	38.33	– 3.55	5
Type-specific assays (strain and isolate number tested)					
EEV-1 (Cascara [RSA1967/03])	0.997	99.8	38.27	– 3.33	1
EEV-2 (Gamil [RSA1971/06])	0.997	102	38.49	– 3.28	3
EEV-3 (Kaalplaas [RSA1974/06])	0.999	96.2	35.43	– 3.42	13
EEV-4 (Bryanston [RSA1976/03])	0.999	98.3	36.44	– 3.30	5
EEV-5 (Kyalami [RSA1976/06])	0.997	100.9	38.25	– 3.31	2
EEV-6 (Potchefstroom [RSA1991/03])	0.998	95.6	36.7	– 3.43	13
EEV-7 (Northrand /E21/20)	0.987	93	36.8	– 3.50	175

assay consistently detected down to five copies of EEV-4 RNA per reaction. Efficiency rates calculated for the different typing assays based on detection of the dilution series of viral RNA varied between 95.6 and 102% (Table 5), which was reflected by slope values between – 3.28 and – 3.43. All six

of the Seg-2 assays showed linearity (R^2) > 0.99 (Table 5). The detection limit of all of the Seg-2 type–specific assays was down to at least 13 RNA molecules. The EEV-7-specific assay, which was tested on DNA copies showed R^2 of 0.987 with 93% efficiency – 3.5 slope. The limit of detection for

Fig. 1 Standard curves for Ct value against EEV-4 (Bryanston) dsRNA number for Seg-9 RT-PCR. Standard curves generated by plotting Ct values against corresponding log copy number of dsRNA genome of EEV-4 (RSA1976/01) in Seg-9 assay. Each dilution was tested in triplicates

the EEV-7 Seg-2-specific assay was calculated as 175 copies (Table 5). However, further validation using viral RNA is needed to accurately measure its sensitivity compared to the other assays.

Discussion

EEV, an arthropod-borne orbivirus, is capable of undergoing rapid changes due to its segmented dsRNA genome potentially exhibiting both genotypic and phenotypic changes. EE is a mild or subclinical disease of horses which is not listed by OIE. However, the clinical signs of EE are similar to the early stages of the more mild forms of AHS, which is a listed OIE disease. Therefore, in order to implement the appropriate control measures as quickly as possible, it is very important (particularly for AHS) to rapidly distinguish AHSV and EEV infections.

There are seven known distinct serotypes of EEV that can be distinguished in serum neutralisation tests [6]. The “gold standard” method for determination of EEV serotype is currently the virus neutralisation test (VNT), which is labour intensive, time consuming, requires prior virus isolation and adaptation to cell culture and sometimes gives inconclusive results. Moreover, they are also dependent on the availability of reference virus strains (as controls) and reference antisera, which are highly characterised and may therefore be difficult to obtain and can themselves represent a potential biosecurity risk. These assays also require disease-secure laboratory facilities for safe handling the live virus.

Molecular methods like real-time RT-PCR (qRT-PCR) assays offer many advantages including high sensitivity and specificity over conventional RT-PCR for the detection of viral RNAs. Since primer and probe designs can be rapidly shared with colleagues without the need for physical movement of reagents or reference materials, the Seg-9-based species-specific and Seg-2-based serotype-specific qRT-PCR assays for different orbiviruses represent a useful alternative to serological testing. RT-PCR methods also have the potential to replace or reduce the need for the use of experimental animals to make antisera for diagnostic assays. For well-characterised orbiviruses such as BTV, EHDV and AHSV, serotype-specific qRT-PCR diagnostic tests have been successfully developed based on a highly variable genome Seg-2 (encoding the serotype-specific VP2 protein), and in many reference laboratories, these assays have become the first choice for diagnostic testing of ‘suspect’ clinical samples [25, 26, 42]. However, a virus species-specific qRT-PCR assay that was recently developed for EEV targeting, the conserved sequences of Seg-7 has comparatively low efficiency (81%) [35].

In this report, we describe the development and evaluation of single sets of two primers and a probe for an EEV Seg-9-based serogroup-specific assay and Seg-2-based serotype-specific

assays for each of the EEV serotypes (Table 2). These assays provide a complete set of effective, sensitive and specific diagnostic tools for the rapid detection and typing of EEV. Both of the assays targeting Seg-9 or Seg-2 detected the reference or field strains (clinical samples and isolates) tested and did not show any cross reactivity with RNA of other related orbiviruses or uninfected controls. The primers and probes have been validated in silico with the published EEV sequences including the Indian EEV-4 strain detected in Pune [43].

The Seg-2 based serotype-specific assays exhibited a high level of sensitivity and specificity showing no evidence of cross-amplification between heterologous EEV serotypes. The detection of EEV RNA from a wide range of reference and field strains, in cell culture supernatants, diagnostic blood or tissue samples indicated that they could be used for molecular diagnosis, surveillance and molecular epidemiology studies of EEV.

The reaction conditions for the group-specific and type-specific EEV assays were optimised to maximise sensitivity using the same cycling conditions making it easier to perform multiple assays at one time. The samples tested in triplicates in different runs showed good reproducibility (99.5 to 99.9%). Both group-specific (Seg-9) and type-specific assays (Seg-2) detected very low concentrations of viral dsRNA (in some cases down to 1 copy per reaction) confirming their sensitivity for detection of EEV even in animals with only a low-level viraemia (Table 5).

The sensitivity and reliability of qRT-PCR assays for detection and typing of BTV and other orbiviruses have previously been demonstrated [25, 26, 44–46]. The diagnostic specificity of the EEV assays described here relates primarily to their ability to detect homologous EEV types and an inability to detect heterologous types or heterologous orbiviruses. However, due to the limited number of EEV isolates that are available, further validation of these assays with additional field strains would be beneficial. In view of the potential for the emergence of novel variant strains of these viruses (that may have changes in assay target regions) periodic re-evaluation and if necessary the redesign of these assays may be required. The authors have the experience with related virus (BTV) where similar refinements have been necessary for BTV type-specific assays in order to maintain their specificity and sensitivity for use in different geographic locations and ecosystems [25].

The group- and type-specific assays described here were used to test and type all of the equine orbiviruses that are available in ORC at the Pirbright Institute. Both AHSV and EEV share the same vectors and have comparable geographic distributions. Therefore, in endemic areas like sub-Saharan Africa, where AHSV and EEV co-circulate, the assays described here provide an important tool for the efficient, reliable and rapid detection and differentiation of the causative agents of these equine diseases [4]. These assays are currently being used for diagnostic and/or research purposes by the reference laboratories and research groups at The Pirbright Institute along with

other established sero-epidemiological tools for the detection of antibodies against EEV including enzyme-linked immunosorbent assay (ELISA) and sequencing.

In conclusion, these studies confirm the successful initial development of fast, robust and reliable ‘TaqMan’ qRT-PCR-based methods for the detection and identification (typing) of EEV. These methods (particularly if linked to sequencing and phylogenetic studies) could be used for epidemiology studies to improve our understanding of the prevalence, distribution and movements of specific EEV strains.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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