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A duplex real-time reverse transcriptase polymerase chain reaction assay for the detection of St. Louis encephalitis and eastern equine encephalitis viruses

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Abstract

A duplex TaqMan real-time reverse transcriptase polymerase chain reaction (PCR) assay was developed for the detection of St. Louis encephalitis virus (SLEV) and eastern equine encephalitis virus (EEEV), for use in human and vector surveillance. The respective targets selected for the assay were the conserved NS5 and E1 genes of the 2 viruses. Because of the insufficient number of NS5 sequences from SLEV strains in the GenBank database, we determined the sequence of an approximately 1-kb region for each of 25 strains of SLEV to select primers and probes in a conserved region. Our assay has a sensitivity of 5 gene copies (gc)/reaction for EEEV and 10 gc/reaction for SLEV, and its performance is linear for at least 6 $\log_{10}$ gc. The assay is specific and detected all strains of SLEV (69) and EEEV (12) that were tested. An internal control ensures detection of efficient nucleic acid extraction and possible PCR inhibition.
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Keywords: Molecular detection; Real-time RT-PCR; Saint Louis encephalitis virus; Eastern equine encephalitis virus

1. Introduction

Eastern equine encephalitis virus (EEEV) is an arthropod-borne virus belonging to the genus *Alphavirus* in the family Togaviridae. The virus is transmitted by mosquitoes to equines, birds, and humans and causes a febrile disease (including encephalitis) with a significant frequency of fatal outcomes. With an overall case fatality rate of approximately 36% (Deresiewicz et al., 1997), it is the most severe of the arboviral encephalitides. EEEV is a spherical enveloped virus with an 11.5-kb genome consisting of single-stranded positive-sense RNA. The 5′ end of the alphavirus genome encodes for the nonstructural proteins (NSP1-4), and the 3′ end of the genome encodes for the structural proteins (core and envelope proteins). The E1 and E2 proteins, virally encoded surface glycoproteins, are the target of numerous serologic reactions and tests. The E2 protein is the site of most of the neutralizing epitopes (Roehrig, 1986), and the E1 protein contains more conserved cross-reactive epitopes (Dalrymple et al., 1976; Wolcott et al., 1984). Apart from the 6 C-terminal codons, the E1 gene is largely conserved among alphaviruses (Powers et al., 2001), although there is sufficient diversity to allow selection of primers and probes that are specific for the EEEV genome.

St. Louis encephalitis virus (SLEV) is also an arthropod-borne virus. This virus belongs to the Japanese encephalitis (JE) virus serocomplex within the genus *Flavivirus* of the family Flaviviridae. SLEV is endemic in the United States and circulates in a transmission cycle involving *Culex* mosquitoes and passerine birds (Tsai and Mitchell, 1989), with humans and other vertebrates as incidental hosts. Clinical disease caused by SLEV infection can vary from mild symptoms, including febrile illness and headache, to severe disease, including meningitis and encephalitis. Reports of case fatalities range from 5% to

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20% (Tsai and Mitchell, 1989), although the numbers are higher still among the elderly population. The virus has a single-stranded positive-sense RNA genome of approximately 11 kb. The 5′ end of the viral genome consists of genes for the structural proteins, and the 3′ end consists of genes for the nonstructural proteins. The NS5 gene encodes the RNA-dependent RNA polymerase, which is reported as being the most highly conserved of the *Flavivirus* nonstructural proteins (Iacono-Connors et al., 1992; Scaramozzino et al., 2001).

Serology is the gold standard for diagnostic testing of arboviruses. However, serologic tests have certain limitations; cross-reactivity of flaviviral antibodies is problematic in immunoglobulin M capture enzyme-linked immunosorbent assays, and plaque reduction neutralization is time consuming, is cumbersome, and requires biosafety level 3 (BSL-3) containment (Calisher, 1994; Martin et al., 2004; Russell and Dwyer, 2000). The traditional method of virus isolation also has the latter limitations, and in addition, because the period of viremia is generally short, the likelihood of obtaining an isolate is low (Calisher, 1994). Reverse transcriptase polymerase chain reaction (RT-PCR) is a rapid and sensitive method that is being used increasingly as an adjunct to serology for the diagnosis of arboviruses. Case in point is the widespread use of PCR for the detection of West Nile virus (WNV) (Briese et al., 2000; Lanciotti and Kerst, 2001).

Our aim was to develop a duplex TaqMan real-time RT-PCR assay for the detection of EEEV and SLEV to test cerebrospinal fluid (CSF) specimens from patients suspected of having arboviral encephalitis. Because there was no sufficient sequence information on the NS5 region of SLEV strains available in the GenBank database, we determined the sequence of an approximately 1-kb region of the NS5 gene for each of 25 strains. Primers and probes were then selected from conserved sequences in this region.

2. Materials and methods

2.1. Viruses and controls

To construct a positive control for the EEEV assay, we performed conventional RT-PCR using primers EEE-E1-F and EEE-E1-R (Table 1) targeting EEEV strain 69-7836 (a 1969 isolate from New York State). The resultant 146-bp fragment of the E1 gene of EEEV was cloned into the PCR-Blunt II-TOPO plasmid (Invitrogen, Carlsbad, CA). The plasmid (pNT21) was linearized and used to transcribe a control RNA transcript containing the real-time RT-PCR target sequence, using the T7 RiboMax Large Scale RNA Production System (Promega, Madison, WI). The transcript RNAs were subjected to 2 rounds of DNase I digestion and were purified by phenol–chloroform extraction and sepharose chromatography. The purified transcript was quantified by measurement of the absorbance at 260 nm. Ten-fold serial dilutions of genomic EEEV strain 69-7836 viral RNA and the transcript were made, and real-time RT-PCR was performed. A standard curve was constructed, allowing quantitation of the genomic viral RNA. Conventional RT-PCR was performed using primers SLE-NS5-F1 and SLE-

Table 1
Primers and probes used in real-time RT-PCR assays for SLEV and EEEV and for internal control detection and for PCR and sequencing of the NS5 region of genomes of SLEV strains

Name of primer or probe	Sequence (5′→3′)	Nucleotide start	Reference
EEE-E1-F	ACACTAAATTCACCCTAGTTCGAT	11 376	This study
EEE-E1-R	GTGTATAAAATTACTTAGGAGCAGCATTATG	11 522	This study
EEE-E1-Fam	6-FAM-CGAGCTATGGTGACGGTGGTGCA-BHQ1	11 407	This study
SLE-NS5-F1	GGTGGTTCGGGAGCCCTT	8678	This study
SLE-NS5-F2	GGTGGTTCGGGAGCCTTT	8678	This study
SLE-NS5-R2	CACGCCTTTTGGCCAACAA	8616	This study
SLE-NS5-R3	CACGCCTTTTGGTCAACAA	8616	This study
SLE-NS5-Vic1	VIC-CAACCTTTTCTTTGAACACC-MGB	8656	This study
SLE-NS5-Vic2	VIC-CAACCTTTTCTTTGAAGACC-MGB	8656	This study
GFP forward primer	CACCCTCTCCACTGACAGAAAAT	549	Tavakoli et al. (2007)
GFP reverse primer	TTTCACTGGAGTTGTCCCAATTC	470	Tavakoli et al. (2007)
GFP probe	6-FAM-TGTGCCCATTAACATCACCATCTAATTCAACA- TAMRA	525	Tavakoli et al. (2007)
FG1	TCAAGGAACTCCACACATGAGATGTACT	8206	Fulop et al. (1993)
FG2	GTGTCCCATCCTGCTGTGTCATCAGCATACA	9167	Fulop et al. (1993)
Seq-1-F	GGAACTATCATGGAAGTTATGAAG	8486	This study
Seq-1-R	TTGCTTTGAATTCTTCTGGGG	8782	This study
Seq-2-F	CTGGCTGTGGGACTTCG	8715	This study
Seq-2-R	GGTCTGGCTTCTCTGTGAGC	8398	This study
Seq-3-F	CCCCAGAAGAATTCAAAGCAAAGG	8762	This study
Seq-3-R	GGCTTCTCTGTGAGCTTTCC	8393	This study
Seq-4-F	CTGGTTGTGGGACTTTGTTGC	8715	This study

EEE = eastern equine encephalitis; SLE = St. Louis encephalitis. EEEV sequence is from GenBank accession EF568607 (EEEV strain NJ/60). SLEV sequence is from GenBank accession EF158050 (SLEV MSI-7). Nucleotide numbers are given based on the genome of MSI-7, but not all primers perfectly match this strain. GFP sequence is from GenBank accession EU341596 (cloning vector pGFPm-T).

NS5-R1 (Table 1) targeting SLEV strain MSI-7. A plasmid (pNT26) that contained the 63-bp NS5 target fragment was constructed as described above. The SLEV transcript was made and quantified, and using this transcript, genomic RNA was quantified as described above.

EEEV and SLEV cultures were amplified in African green monkey kidney (Vero) cell cultures in a BSL-3 facility. Because EEEV is a Centers for Disease Control and Prevention (CDC) Select Agent (SA), the virus and RNA were handled by SA-approved personnel only in a BSL-3 laboratory with controlled access, certified under protocol number C2005 0318-0344. Powered air purifying respirators were worn at all times when personnel were working with infectious EEEV.

Sixty-nine SLEV strains were cultured in Vero cells (Table 2). Sixty-six of these strains were received by the Arbovirus Laboratory from the World Reference Center of Emerging Viruses and Arboviruses at the University of Texas Medical Branch in Galveston, TX.

Genomic copy numbers of cell culture-propagated EEEV and SLEV genomic RNAs were determined from a standard curve produced from the assay of 10-fold dilutions of purified and quantified EEEV and SLEV RNA transcripts. The RNA from the cell culture-propagated EEEV and SLEV, and the RNA transcripts were assayed with the EEEV E1 and SLEV NS5 primer and probe sets (Table 1). We quantified genomic SLEV and EEEV RNA by performing real-time RT-PCR on serial dilutions of transcript and genomic RNAs and then constructing a standard curve, using serial dilutions of quantified transcript RNA as standards. In addition, a no RT control was performed at a high transcript RNA concentration to ensure that the bulk of the DNA had been digested.

EEEV RNA transcript (500 gene copies [gc]/reaction) was used as the positive control. The advantage of using RNA transcript rather than genomic RNA as the positive control is that the RNA transcript, unlike the genomic EEEV RNA, can be handled in a BSL-2 laboratory that is not SA certified by non–SA-approved personnel; diagnostic testing for EEEV is generally performed in BSL-2 facilities. Genomic MSI-7 RNA (1100 gc/reaction) was used as the positive control for the SLEV assay.

As an internal nucleic acid extraction control and for detection of potential PCR inhibition, each clinical specimen was spiked during the lysis step of the extraction process, with a known quantity of green fluorescent protein (GFP) RNA transcript (2200 gc/μL). After completion of the extraction, a one-tenth dilution of the extracted nucleic acid was analyzed by a 1-step real-time RT-PCR with a primer and probe set specific for GFP (Table 1). A positive result, with a Ct value falling within the defined range (34–38), from the GFP assay confirmed the successful extraction of nucleic acid.

Severe acute respiratory syndrome (SARS) coronavirus Urbani strain was obtained from the CDC, cytomegalovirus (CMV) DNA (0.01 μg/μL) was purchased from Sigma-Aldrich (St. Louis, MO), and adenovirus and human herpes

Table 2
SLEV strains used in this study

Strain name	Acc. Id.	Strain name	Acc. Id.	Strain name	Acc. Id.
GML 902612	EF158064	BFS 508	N/A	798-55	N/A
PAN AR 902745	EU076713	Fort Washington	N/A	BeAn 208331	N/A
TNM-4-711K	EF158065	BeAn 421498	N/A	BeAn 211991	N/A
GHA-3	EF158066	BeAn 423728	N/A	BeAn 212371	N/A
BeAn 247377	EF158067	BE H 203235	N/A	TD6-4G	N/A
BeAr 23379	EF158048	BFN 1324	N/A	P17787	N/A
904.3	EF158049	Hubbard	N/A	L69-5121.05	N/A
CorAn 9275	EF158068	BeAn 261207	N/A	TEX 16017	N/A
72 V 4749	EF158069	GML 902613	N/A	TEX M6	N/A
MSI-7	EF158050	GML 902981	N/A	VP 34	N/A
Parton	EF158070	GML 902984	N/A	TH 4-9f	N/A
65 V 310	EF158059	GML 902991	N/A	Texas 1955	N/A
GML 903797	EF158060	GML 903050	N/A	TEX 1198	N/A
GMO 94	EF158051	BV7 (GML 900968)	N/A	VP 7	N/A
CorAn 9124	EF158063	75256 PG3	N/A	3082 = B32	N/A
V 2380-42	EF158052	2088	N/A	2785 = B37	N/A
BeAn 246262	EF158053	2234	N/A	M04 (U0476)	N/A
75 D 90	EF158054	6943 STL	N/A	GML 903369	N/A
69 M 1143	EF158061	61V 2416	N/A	V 4285 (M 59)	N/A
TBH 28	EF158055	68V 1587	N/A		
TRVL 9464	EF158056	BeAn 421297	N/A		
78 A 28	EF158057	75V 14868	N/A		
FL 79-411	EF158062	BeAn 401517	N/A		
Kern-217	EF158058	75V 6507	N/A		
CoAv 608	EU099355	75V 2533	N/A		

N/A = sequence information in the NS5 region was not available for this strain in the GenBank database; Acc. Id. = GenBank accession identification number.

virus 6 (HHV-6) DNA were obtained from ATCC (Manassas, VA). Virus suspensions of coxsackievirus A9 (Cox A9), coxsackievirus B12 (Cox B12), echovirus 9 (Echo 9), human coronavirus 229E (HCoV 229E), human metapneumovirus (hMPV), influenza A-H1 virus (FluA H1), influenza A-H3 virus (FluA H3), influenza B virus (FluB), respiratory syncytial virus (RSV), human rhinovirus (HRV), Epstein–Barr virus (EBV), herpes simplex viruses (HSV) 1 and 2, and varicella zoster virus (VZV) were obtained from the Proficiency Testing Laboratory and Virus Reference and Surveillance Laboratory at the Wadsworth Center, Albany, NY. Dengue virus serotypes 1 to 4 (DENV1–4), western equine encephalomyelitis (WEE) virus, Highlands J virus, WNV, Murray Valley encephalitis (MVE) virus (MK6684), JE virus (Nakayama-NIH), yellow fever (YF) virus (YF 17D), Powassan (POW) virus 64-7062 (representative of POW virus lineage I), and DT-SPO (representative of POW virus lineage II) were obtained from the Arbovirus Laboratory at the Wadsworth Center. Bacterial cultures of *Corynebacterium xerosis*, group A *Streptococcus*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Neisseria meningitidis* groups B, C, and Y, *Neisseria subflava*, *Streptococcus sanguis*, and *Streptococcus pneumoniae* serotypes 10A, 11A, and 18F were obtained from the Bacteriology Department at the Wadsworth Center.

2.2. Nucleic acid extraction

Nucleic acid was extracted from specimens using the NucliSens miniMAG or easyMAG system (bioMérieux, Durham, NC). Two hundred fifty microliters of each specimen were added to 2 mL of lysis buffer. Five microliters of GFP transcript (2100 gc/μL) were spiked into the lysed sample. After miniMAG or easyMAG extraction, the nucleic acid was eluted in 50 or 55 μL of elution buffer, respectively.

2.3. Real-time RT-PCR

Real-time RT-PCR for the detection of SLEV and EEEV viruses was performed using primers and probe developed in-house (Table 1). The EEEV probe was labeled with the reporter 6-carboxyfluorescein (6-FAM) at the 5′ end and a black hole quencher (BHQ1) (Operon, Huntsville, AL) at the 3′end. The 2 SLE probes were each labeled with the reporter VIC™ at the 5′ end and a minor groove binder (MGB) (Applied Biosystems, Foster City, CA) at the 3′end. Amplification was carried out in a 25-μL volume reaction using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen), 0.5 μL of 5× 5-carboxy-X-rhodamine (ROX) reference dye, 400 nmol/L each of 4 SLEV primers, 500 nmol/L each of 2 EEEV primers, 75 nmol/L each of 2 SLEV probes, and 200 nmol/L EEEV probe. The reactions were incubated at 48 °C for 30 min, followed by 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. A separate real-time RT-PCR for the detection of GFP was performed with the primers and probes listed in Table 1. The GFP probe was labeled with the reporter 6-FAM at the 5′ end and the quencher 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3′ end. The reaction consisted of universal buffer (Applied Biosystems), the forward and reverse primers at 900 nmol/L each, and 250 nmol/L probe (Tavakoli et al., 2007). The reaction conditions were as above. PCR reactions were performed using an ABI 7500 instrument (Applied Biosystems).

2.4. PCR and sequence determination of SLEV strains

Nucleic acid from SLEV cultures was extracted with the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI). RNA was transcribed to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). PCR, to amplify a portion of the NS5 region of SLEV, was performed essentially as reported previously (Fulop et al., 1993; Table 1), except that an initial incubation time of 95 °C for 15 min was used to activate the HotStar Taq polymerase (QIAGEN, Valencia, CA). PCR products were analyzed by agarose gel electrophoresis and were stained by ethidium bromide. The PCR products, which were approximately 958 bp in size, were extracted from the gel and spun through an Ultrafree-DA Filter Device (Millipore, Billerica, MA). Sequencing reactions of the PCR products were performed with multiple primers shown in Table 1. The concentration of each primer was 3.2 pmol, and sequencing reactions were performed at the Wadsworth Center Molecular Genetics Core facility on an automated DNA sequencer Model 3100 (Applied Biosystems). The obtained sequences were submitted to the GenBank database (see Table 2 for accession numbers).

3. Results

Sequence information was obtained for approximately 950 bp of the NS5 gene from each of 25 different strains of SLEV (Table 2). The sequences were aligned with the MEGA 4 software (Tamura et al., 2007). Regions that showed minimal sequence variation were chosen as candidate regions for primer/probe target selection. The same process was repeated for the E1 region from sequences from multiple strains of EEEV found in the GenBank database. Primers and probes were selected with the Primer Express software (Applied Biosystems) (Table 1). For detection of the maximum number of SLE strains, we selected 2 forward primers, 2 reverse primers, and 2 probes because we were unable to find 1 set of primers and probe that were 100% homologous with all strains. Each of the forward primers, reverse primers, and probes had 1 mismatch.

Because of the SA status of EEEV, a positive control for the assay was constructed that could be used in a BSL-2 laboratory by non–SA-approved personnel, thereby facilitating the assay's diagnostic application. The control was an RNA transcript containing a 146-bp portion of the envelope-coding gene of EEEV. This control was also used for the quantification of genomic EEEV RNA. The SLEV

transcript, which was used for the quantification of SLEV genomic RNA, was prepared from a recombinant plasmid containing a 63-bp portion of the NS5-coding gene of SLEV strain MSI-7.

After construction and quantification of the controls for the assay, various real-time PCR kits from several manufacturers were evaluated for the optimization of our duplex assay. These were TaqMan One-Step RT-PCR kit (Applied Biosystems), BioRad iScript One-Step RT-PCR kit for probes (Bio-Rad Laboratories), FailSafe Probes Real-Time PCR Optimization kit (Epicentre Biotechnologies), SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen), Quantitect Multiplex PCR kit (QIAGEN), Quantitect Probe RT-PCR kit (QIAGEN), and LightCycler FastStart DNA Master Hybridization Probes kit (F. Hoffmann-La Roche, Nutley, NJ). Because the SuperScript III Platinum One-Step Quantitative RT-PCR System presented a combination of the lowest Ct and highest ΔRn values (data not shown), we optimized the assay using this kit.

Primer optimization was performed with primer concentrations ranging from 100 to 1000 nmol/L, and probe optimization was performed with probe concentrations ranging from 50 to 400 nmol/L. For a TaqMan assay, optimal performance is achieved by selecting primer/probe concentrations that provide the lowest Ct and the highest ΔRn for a fixed amount of target template (Cirino et al., 2007). The Ct and ΔRn data taken together show that the optimal SLEV primer concentrations were 400 nmol/L each, and the optimal SLEV probe concentrations were 75 nmol/L each. Optimal EEEV primer concentrations were 500 nmol/L each, whereas the optimal EEEV probe concentration was 200 nmol/L.

Serial dilutions of the SLEV and EEEV transcripts were made, and the duplex real-time RT-PCR assay was performed. The assay can detect a range between 5 and 5×10^6 gc of EEEV and a range between 10 and 3×10^6 gc for SLEV. We were consistently able to detect 5 gc of EEEV target (performed 12 times) and 10 gc of SLEV target (performed 14 times). The assay range and sensitivity of the duplex assay, when compared with the SLE and EEE assays performed separately as singleplex assays, gave similar results, indicating that there is no negative interference between the primer probe sets of the 2 assays (data not shown).

To determine the specificity of the assay, we performed the assay using high concentrations (for each virus, approximately 10^6 gc) of nucleic acid from the following organisms: Cox A9, Cox B12, Echo 9, HCoV 229E, hMPV, FluA H1, FluA H3, FluB, RSV, HRV, SARS coronavirus, adenovirus, CMV, EBV, HHV-6, HSV 1 and 2, VZV, WEE virus, Highlands J virus, DENV1–4, POW virus (representatives of lineage I and II), WNV, YF virus, MVE virus, JE virus, *C. xerosis*, group A *Streptococcus*, *H. influenzae*, *H. parainfluenzae*, *N. meningitidis* groups B, C, and Y, *N. subflava*, *S. sanguis*, and *S. pneumoniae* serotypes 10A, 11A, and 18F. No cross-reactivity was observed between the EEEV and SLEV primer/probe sets and any of the organisms selected in the specificity panel. PCR results for the panel were all negative; therefore, the assay was specific upon testing a wide range of pathogens.

EEEV is the sole species in the EEEV antigenic complex and is divided into North American and South American antigenic varieties based on hemagglutination inhibition tests. On the basis of nucleotide sequencing and serologic tests, including plaque reduction neutralization tests, 4 major lineages/clusters (clusters 1–4) with distinct antigenic subtypes have been identified (Brault et al., 1999). Serial dilutions were made of the RNA from representatives of each cluster (Table 3). We then performed the singleplex real-time RT-PCR assay for the detection of EEEV on various RNA dilutions to determine whether nucleic acid from the 12 different strains would be amplified by the real-time RT-PCR reaction. Real-time RT-PCR was performed on the various nucleic acid dilutions in a BSL-3 laboratory. Results showed that all 12 strains were detected and amplified by the EEEV primer/probe set.

Nucleic acid was extracted from each strain of amplified SLEV, and dilutions were made and tested in the duplex real-time RT-PCR assay. All strains were detected and amplified. Because we were unable to obtain patient specimens that were positive for either SLEV or EEEV, we spiked SLEV culture and EEEV transcript, at each of 3 dilutions, into negative CSF specimens. Appropriate dilutions of SLEV strain MSI-7 culture were made to obtain the high (9.9×10^3 gc/250 μL CSF equivalent to 9000 gc/reaction), medium (990 gc/250 μL CSF equivalent to 90 gc/reaction), and low (99 gc/250 μL CSF equivalent to 9 gc/reaction) concentrations. Similarly, a range of nucleic acid concentrations that included high (5.5×10^3 gc/250 μL CSF equivalent to 5000 gc/reaction), medium (5.5×10^2 gc/250 μL CSF equivalent to 500 gc/reaction), and low (550 gc/250 μL CSF equivalent to 50 gc/reaction) were selected for the EEEV

Table 3
EEEV strains used in this study

Strain (Acc. Id.)	Year and location (state) of isolation	Classification
39030199	2003, New York	Cluster 1
04002727	2004, New York	Cluster 1
US03-1974v1	2003, New Jersey	Cluster 2
US03-1977v1	2003, New Jersey	Cluster 2
04002073	2004, New York	Cluster 3
US02-4341v1	2002, Florida	Cluster 3
36040401	2004, New York	Cluster 4
US03-2767	Unknown	Cluster 4
NY69-7836 (EF594003)	1969, New York	NC
36030127	2003, New York	NC
EEE71-20686 (EF594005)	1971, New York	NC
EEE74-39100 (EF594015)	1974, New York	NC

Acc. Id. = accession identification numbers from the GenBank database are presented in parentheses where available; NC = not classified.

transcript. These dilutions were then spiked into CSF that had previously tested negative for EEEV and SLEV. Forty negative samples were either not spiked or spiked with 1 of 3 concentrations of SLEV culture or of EEEV transcript in various combinations (Table 4). All 40 blinded samples were also spiked with a known amount of GFP RNA transcript (2200 gc/μL), which acts as the internal control. RNA was extracted from the specimens using the NucliSens easyMAG system. GFP was then detected in the sample via real-time RT-PCR to determine extraction efficiency and the presence of inhibitors (Table 4).

For each of the blinded samples, the high, medium, and low gc samples could all be detected indicating minimal loss in sensitivity after nucleic acid extraction (Table 4). All of the negative extraction controls had Ct values of >45, indicating that false positives were not detected and that no cross-contamination had occurred during extraction of the samples. Also, all real-time RT-PCR experiments included positive EEEV (500 gc/reaction) and SLEV (1100 gc/reaction) controls. Both EEEV and SLEV positive controls gave Ct values within the acceptable ranges for each (29–32 and 31–34, respectively). The Ct values for GFP for all the samples were within the acceptable range (34–38), with the exception of 1 sample (Table 4, sample with Ct of 38.66) that showed slight inhibition. The EEEV spike and SLEV spike were both detected for this sample. In general, if a clinical specimen showed a higher than acceptable GFP Ct value, the recommendation would be to repeat the extraction and real-time RT-PCR.

In a separate experiment, 30 different negative CSF specimens were spiked with 1 of 3 concentrations of EEEV culture (5×10^4, 5×10^3, and 5×10^2 gc/250 μL CSF equivalent to 5000, 500, and 50 gc/reaction). After nucleic acid extraction with an internal GFP control, we performed real-time RT-PCR for the detection of EEEV. In addition, we performed the singleplex real-time RT-PCR assay for the detection of EEEV on RNA extracts from 5 uninfected mosquito pools and on RNA extracts from 5 EEEV-infected mosquito pools ($\log_{10}$ pfu ranging from 0.4 to 4.4/0.1 mL). The assay correctly identified all specimens, showing that it is suitable for use on vector specimens as well as viral culture.

4. Discussion

Human infections by SLEV and EEEV are relatively rare, although EEEV infection of equines occurs regularly. Nevertheless, severe morbidity and mortality associated with these 2 arboviruses make them important public health concerns. The surveillance of virologic activity in natural hosts, as well as of disease in humans and horses, is essential for early detection of outbreaks and implementation of vector control measures. Molecular detection by RT-PCR is an ideal method for detection of these viruses in hosts because it is rapid, sensitive, specific, reproducible, and amenable to automation. RT-PCR has been used for the detection of arboviruses at the group level (Bronzoni et al., 2004; Pfeffer et al., 1997; Sanchez-Seco et al., 2001; Scaramozzino et al., 2001) and for specific detection of individual arboviruses (Lambert et al., 2003; Lanciotti and Kerst, 2001; Lee et al., 2002; Linssen et al., 2000; O'Guinn et al., 2004), as well as for a sequential detection at both levels (Bronzoni et al., 2005).

In terms of geographic distribution, EEEV is found mainly in the eastern half of the United States, with most transmissions occurring near freshwater hardwood swamps in states along the Atlantic seaboard and the Gulf Coast, and in the Great Lakes region. Between 1964 and 2004, the greatest numbers of human cases in the United States occurred in Florida, Georgia, Massachusetts, and New Jersey (http://www.cdc.gov/ncidod/dvbid/arbor/eeefact.htm). SLEV, in contrast, is found throughout the 48 contiguous states, but periodic epidemics have only been documented in the Midwest and the Southeastern United States (including

Table 4
Average Ct values obtained when the EEEV, SLEV, and GFP real-time RT-PCR assays were performed on extracts of CSF specimens spiked with SLEV culture and EEEV transcript

Sample	Virus/transcript spike	Average Ct for viral assay	Ct range for viral assay	CV for viral assay	Average Ct for GFP assay	Ct range for GFP assay	CV for GFP assay
NTC	–	≥45	≥45 to ≥45	0	≥45	≥45 to ≥45	0
EEEV spike							
	Negative	≥45	≥45 to ≥45	0	35.01	34.89–35.11	0.2%
	Low	39.22	37.55–41.3	2.85%	35.30	34.87–35.87	0.97%
	Medium	36.41	35.44–38.81	2.47%	35.65	34.92–38.66	3.04%
	High	32.21	31.57–32.62	0.83%	35.52	34.92–35.97	1.09%
SLEV spike							
	Negative	≥45	≥45 to ≥45	0	35.21	34.87 to 35.87	0.98%
	Low	39.62	38.04 to 41.75	2.44%	35.27	34.92 to 35.97	1.09%
	Medium	34.07	33.48 to 34.78	0.75%	35.27	34.96 to 35.69	0.77%
	High	29.67	29.39 to 30.08	0.63%	35.73	34.95 to 38.66	3.02%

NTC = no template control. The high, medium, and low spikes for SLEV were 9000, 90, 9 gc/reaction, respectively; the high, medium, and low spikes for EEEV were 5000, 500, and 50 gc/reaction, respectively. The average numbers are from 10 assays performed in duplicate for virus detection and performed singly for GFP detection. CV = coefficient of variation.

Florida) (http://www.cdc.gov/ncidod/dvbid/sle/index.html; Wells et al., 1990). A molecular assay that detects both SLEV and EEEV, thus, has considerable application for patients suspected of having arboviral encephalitis who reside in these areas, as well as travelers who could have come into contact with infected mosquitoes.

We report the development of a duplex TaqMan real-time RT-PCR assay for the detection of SLEV and EEEV. The targets for the PCR assay are sited within conserved regions of the genome, namely, the E1 gene of EEEV and the NS5 gene of SLEV. The assay is intended for patient CSF samples but can easily be used for vector surveillance. We found the optimal PCR kit for this assay to be the SuperScript III Platinum One-Step Quantitative RT-PCR System. The linear assay range is 5 to 5×10^6 gc/reaction for EEEV and 10 to 3×10^6 gc/reaction for SLEV. The sensitivity of the assay is 5 gc/reaction for EEEV and 10 gc/reaction for SLEV. The assay is specific, in that the primers and probes did not cross-react with any of the organisms in the specificity panel that we used. The assay detected all of the strains of SLEV (69 strains) and EEEV (12 strains) that we tested. We compared our assay with a previously published real-time TaqMan RT-PCR assay for the detection of SLEV, which targets the envelope gene (Lanciotti and Kerst, 2001). Although the 2 assays were similar in sensitivity and specificity, the previously published assay did not detect all SLEV strains; 4 of the 69 SLEV strains in Table 2 (CorAn 9275, CorAn 9124, GML 903797, and GML 903369) were not detected (data not shown). This is not surprising given that the primers and probe had multiple mismatches when aligned with the genome sequence of these strains; as many as 10 mismatches in total occurred for each of strains CorAn 9275 and CorAn 9124. In our SLEV assay, the primers and probes were selected to enable the detection of a greater variety of strains by targeting the conserved NS5 region.

The spiking experiments in patient CSF specimens show that our extraction procedure is efficient, that is, there is little loss of virus or internal control during the process, and that the assay correctly identifies the EEEV or SLEV genome present in the specimen. In addition, many of the samples had both viruses present, in some cases, at high concentration. Nevertheless, detection of both viral genomes occurred just as efficiently as if there was no competitor virus present (Table 4), indicating that neither assay was inhibited.

The use of the GFP internal control ensures that an extraction and PCR inhibition control are included in the assay. It should be noted that, in our experience, after having tested well more than 1000 CSF specimens, CSF specimens rarely (<1%) exhibit significant PCR inhibition, whereas a larger number of plasma and serum specimens show inhibition. We would, therefore, recommend that if plasma or serum specimens are tested, the nucleic acid extracts from the specimens are not diluted before performing the GFP real-time RT-PCR assay to detect inhibition more accurately because the predilution may overcome some of the inhibitory effect.

Our data show that the duplex assay is highly specific, reproducible, and sensitive; furthermore, with a turnaround time of approximately 5 h (including the nucleic acid extraction step), it is a rapid method of detecting EEEV and SLEV RNA in patient samples. Additionally, it is suitable for high-throughput approaches useful in surveillance activities.

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