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# A duplex real-time reverse transcriptase polymerase chain reaction assay for the detection of California serogroup and Cache Valley viruses<sup>☆</sup>

Heng Wang<sup>a,1</sup>, Seela Nattanmai<sup>a,1,2</sup>, Laura D. Kramer<sup>a,b</sup>,  
Kristen A. Bernard<sup>a,b</sup>, Norma P. Tavakoli<sup>a,b,\*</sup>

<sup>a</sup>Wadsworth Center, New York State Department of Health, Albany, NY 12201, USA

<sup>b</sup>Department of Biomedical Sciences, School of Public Health, University at Albany, Albany, NY, USA

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## Abstract

A duplex TaqMan real-time reverse transcriptase polymerase chain reaction (PCR) assay was developed for the detection of California (CAL) serogroup viruses and Cache Valley virus (CVV), for use in human surveillance. The targets selected for the assay were the sequences encoding the nucleocapsid protein of CAL and the G1 glycoprotein of CVV. Conserved regions were selected by aligning genetic sequences from various strains available in the GenBank database. Primers and probes were selected in conserved regions. The assay sensitivity was 75 gene copies (gc)/reaction for CAL serogroup viruses and 30 gc/reaction for CVV. The performance of the assay was linear over at least 6 log<sub>10</sub> gc. The assay was specific, given that it did not cross-react with a variety of pathogens. It did, however, detect 11 viruses within the CAL serogroup and 12 CVV isolates. The use of an internal control ensured that possible inefficiency in nucleic acid extraction or PCR inhibition would be detected.

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**Keywords:** Molecular detection; Real-time RT-PCR; California serogroup viruses; Cache Valley virus

## 1. Introduction

California (CAL) serogroup viruses and Cache valley virus (CVV) are arthropod-borne viruses belonging to the genus *Bunyavirus*, in the family *Bunyaviridae*. Bunyaviruses circulate in specific transmission cycles consisting of a number of vertebrate hosts and preferred mosquito vectors including several species of *Aedes*. Humans are incidental hosts in whom both groups of viruses can cause central nervous system (CNS) diseases, particularly encephalitis and meningitis, although confirmed cases occur infrequently.

CAL serogroup includes the following viruses: La Crosse (LACV), Jamestown Canyon (JCV), Snowshoe hare

(SSHV), Trivittatus (TVTV), Keystone, Tahyna (TAHV), Inkoo (INKV), and CAL encephalitis (CEV) viruses. Before 2002, members of the CAL serogroup, LACV in particular, constituted the leading cause of arboviral encephalitis in the United States (Romero and Newland, 2006). The most pathogenic member, and the most common cause of reported illness in this group, remains LACV. Children are particularly susceptible to La Crosse encephalitis (Balkhy and Schreiber, 2000; Romero and Newland, 2006). The majority of LACV infections are subclinical or lead to mild symptoms. More serious cases result in encephalitis with symptoms of severe bifrontal headache, fever, vomiting, and lethargy progressing to seizures and coma (Balkhy and Schreiber, 2000; McJunkin et al., 1997, 2001). Symptoms of fever, focal seizures, and lateralizing epileptiform discharges can cause a misdiagnosis of herpes simplex encephalitis (McJunkin et al., 2001). TAHV and INKV viruses, in general, result in fever and in severe cases, CNS involvement can also occur (Demikhov et al., 1991). JCV affects mainly adults and causes a febrile illness and mild CNS disease (Srihonge et al., 1984).

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\* Corresponding author. Empire State Plaza, PO Box 509, Albany, NY 12201, USA. Tel.: +1-518-402-5210; fax: +1-518-486-2693.

E-mail address: [norma.tavakoli@wadsworth.org](mailto:norma.tavakoli@wadsworth.org) (N.P. Tavakoli).

<sup>1</sup> Heng Wang and Seela Nattanmai contributed equally to this work.

<sup>2</sup> Present address: Albany Medical Center, Albany, NY.

CVV is associated with encephalitis and meningitis in humans, but reported cases are rare (Campbell et al., 2006; Sexton et al., 1997). Preliminary evidence suggests that CVV can cause congenital defects in humans, but this assertion needs to be further investigated (Calisher and Sever, 1995). CVV is the causal agent of congenital malformations and fetal death in sheep (Chung et al., 1990; Edwards, 1994) and has also been found to infect cattle, horses, and white-tailed deer (Blackmore and Grimstad, 1998; McLean et al., 1987). The transmission cycle of CVV includes small mammals, such as rabbits and mosquitoes. The virus has been isolated from multiple species of anopheline or culicine mosquitoes, including *Anopheles quadrimaculatus* and *Culiseta inornata* (Calisher et al., 1986a).

Reverse transcriptase polymerase chain reaction (RT-PCR) is a rapid and sensitive method that is being used increasingly as a supplement to serology, for the diagnosis of arboviruses. The technique has wide application for clinical diagnostics as well as surveillance purposes. Conventional PCR methods for the amplification of bunyavirus genomes have previously been reported (Campbell and Huang, 1999; Dunn et al., 1994; Kuno et al., 1996).

Our aim was to develop a duplex TaqMan real-time RT-PCR assay for the detection of CAL serogroup viruses and CVV, for testing of cerebrospinal fluid (CSF) specimens from patients suspected of having arboviral encephalitis. In addition, the assay that we designed can be used to perform surveillance in mosquito vectors. The advantages of a multiplexed assay are that cost, as well as hands-on time, is reduced and patient samples are conserved.

Bunyaviruses have a negative-strand trisegmented genome consisting of large (L), medium (M), and small (S) RNA segments. The M RNA segment encodes a single open reading frame (ORF) that is translated into the structural glycoproteins G1 and G2 and a nonstructural protein of unknown function (Fazakerly et al., 1988; Jacoby et al.,

1993; Shope et al., 1981). In a study comparing the G1 glycoprotein-coding sequences of a number of CVV isolates with sequences of a prototype virus, an overall identity ranging from 91% to 99.4% was reported (Brockus and Grimstad, 2001), indicating that the region is well conserved. We chose the portion of the M segment encoding the G1 glycoprotein as a candidate region for selection of primers and probe for the CVV assay.

The S RNA segment of bunyaviruses codes for the nucleocapsid (N) protein and a nonstructural protein, NS<sub>S</sub>; these 2 proteins are encoded in overlapping ORFs (Fuller et al., 1983). Among the N ORF sequences of the CAL serogroup viruses, the nucleotide sequence identities range from 72.0% to 97.3% (Huang et al., 1996). This is marginally higher than the nucleotide sequence identities of the M RNA segments of these viruses, which range from 65.4% to 96.9% (Campbell and Huang, 1999). We, therefore, targeted the conserved N ORF sequences for selection of primers and probe for the CAL serogroup assay.

## 2. Materials and methods

### 2.1. Viruses and controls

To construct a quantitation standard for the CAL serogroup virus assay, we performed conventional RT-PCR using primers CE-NC-F1 and CE-NC-R2 (Table 1) that targeted LACV. The resultant 60-bp sequence of the N ORF was cloned into the PCR-Blunt II-TOPO plasmid (Invitrogen, Carlsbad, CA). The plasmid (pNT27) was linearized and used to transcribe a control RNA transcript containing the real-time RT-PCR target sequence. Transcription was performed 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

Table 1

Primers and probes used in real-time RT-PCR assays for CAL serogroup viruses and CVV and for internal control detection

Name of primer or probe	Sequence (5'→3')	Nucleotide start	Reference
CE-NC-F1	GTGTTTATGATGTCGCATCA	94	This study
CE-NC-F2	GTTTCTATGATGATGCATCC	94	This study
CE-NC-R1	CATATACCCTGCATCAGGATCAA	153	This study
CE-NC-R2	CACAAACCCTGCATCTGGATCAA	153	This study
CE-NC-FamMGB	Fam-CAGGTGCAAATGGA-MGB	116	This study
CV-Mex-F	GCACTCTGGCAGGCAGGA	2220	This study
CV-Mex-R	GACGTCTGTTAAGAAGCAAGTTGAGTTT	2520	This study
CV-G1-F	CCAATGCAATTCAGGGCAGT	2246	This study
CV-G1-R	TGAGTCACCACATGCTGTAAGGT	2358	This study
CV-G1-VicMGB	Vic-AAGAATGCCATAATGCA-MGB	2273	This study
GFP forward primer	CACCCTCCTCACTGACAGAAAAT	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)

LACV sequence is from GenBank accession K00610 (LACV S segment). Nucleotide numbers are given based on the K00610 accession, but CE-NC-F2 and CE-NC-R2 do not perfectly match the sequence of the isolate represented by this accession. CVV sequence is from GenBank accession AF186243 (CVV strain 807270 M segment). Nucleotide numbers are given based on the AF186243 accession, but the CV-Mex-R primer does not perfectly match the sequence of strain 807270. GFP sequence is from GenBank accession EU341596 (cloning vector pGFPm-T).

using the RNA 6000 Pico kit (Agilent Technologies, Santa Clara, CA) on the Agilent 2100 Bioanalyzer. Ten-fold serial dilutions of genomic LACV 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 CVV-Mex-F and CVV-Mex-R (Table 1) targeting CVV (strain 62-7364). With the method described above, a plasmid (pNT28) was constructed, which contained the 301-bp G1 glycoprotein-encoding sequence that spanned the real-time PCR target region for the CVV real-time RT-PCR assay. The CVV transcript was made and quantified, and, with the use of this transcript, genomic RNA was quantified as described above.

CVV isolates were obtained during routine surveillance for West Nile virus (WNV). Briefly, mosquitoes were pooled in groups of 50 females of the same species, homogenized in 1.0-mL mosquito diluent (phosphate-buffered saline with 20% fetal bovine serum, penicillin/streptomycin, and Fungizone), and centrifuged for 2 min at  $13\,000 \times g$ . One hundred microliters of the supernatant were placed on a fresh monolayer of African green monkey kidney cells (Vero). The cell cultures were monitored for 7 days for the presence of cytopathic effect (CPE). Isolates were made from CPE-positive cultures, screened by RT-PCR using *Bunyavirus* generic primers, and identified by sequencing (Ngo et al., 2006).

The CAL serogroup viruses were historic viral cultures that were previously prepared as described (Campbell and Huang, 1995) and were stored in our laboratory. We sequenced a region of approximately 200 bp of the S RNA fragment of each of the viruses to confirm their identities. Accession numbers of the isolates having the highest identity with isolates used in our study are presented in Table 2.

Genome copy numbers of cell culture-propagated LACV and CVV genomic RNAs were determined from a standard curve produced from the assay of 10-fold dilutions of purified and quantified LACV and CVV RNA transcripts. The RNA from the cell culture-propagated LACV and CVV and the RNA transcripts were assayed with the CAL serogroup virus NC and the CVV G1 primer and probe sets (Table 1). We quantified genomic LACV and CVV RNAs 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.

Genomic LACV RNA (approximately 300 gene copies [gc]/reaction) was used as the positive control for the CAL serogroup virus detection assay. Genomic CVV RNA (approximately 1000 gc/reaction) was used as the positive control for the CVV assay.

As an internal nucleic acid extraction control and for detection of potential PCR inhibition, each clinical specimen

Table 2  
CAL serogroup viruses used in this study

Virus	Complex	Isolate	GenBank accession	Date of isolation	Reference
LACV	CE	67.8109	K00610	1984	NA
San Angelo virus	CE	NA	U47139	1982	NA
Lumbo virus	CE	NA	X73468	1983	NA
CEV	CE	65-7210	U12797	1983	NA
TAHV	CE	NA	U47142	NA	NA
SSHV	CE	NA	EU294510	1981	NA
JCV	Mel	NA	U12796	1982	NA
INKV	Mel	SWAR 83-161	U47138	1995	Huang et al. (1996)
Melao virus	Mel	NA	U12802	1985	NA
Keystone virus	Mel	Pr96144-153	U12801	1983	NA
TVTV	TVT	Eklund	AF123491	1987	Campbell and Huang (1995)

Accession numbers for INKV and TVTV are for the isolates used in this study. Accession numbers for all other CAL serogroup viruses are for isolates with the highest identity with isolates used in this study. NA = not available; CE = CAL encephalitis; Mel = Melao; TVT = Trivittatus.

was spiked during the lysis step of the extraction process with a known quantity of green fluorescent protein (GFP) RNA transcript (2200 gc/ $\mu$ L) (Hull et al., 2008). After completion of the extraction, the 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 for the GFP assay, with a Ct value falling within the defined range (35–38), confirmed the successful extraction of nucleic acid.

Severe acute respiratory syndrome (SARS) coronavirus Urbani strain was obtained from the Centers for Disease Control and Prevention (CDC), cytomegalovirus (CMV) DNA (0.01  $\mu$ g/ $\mu$ L) was purchased from Sigma-Aldrich (St. Louis, MO), and adenovirus and human herpes virus 6 (HHV-6) DNAs were obtained from ATCC (Manassas, VA). Virus suspensions of coxsackievirus A9 (Cox A9), echovirus 9 (Echo 9), human coronavirus 229E (HCoV 229E), human metapneumovirus (hMPV), 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 (HJV), WNV, St. Louis encephalitis virus (SLEV) (strains MSI-7 and Kern), Powassan (POW) virus 64-7062 (representative of POW virus lineage I), and DTV-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.

Negative extraction control for the assay consisted of supernatant from uninfected HEL cells cultured in Eagle's minimum essential medium- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (EMEM-HEPES).

## 2.2. Nucleic acid extraction

Nucleic acid was extracted from specimens using the NucliSens miniMAG or easyMAG system (bioMerieux, Durham, NC). Two hundred fifty microliters of each specimen were added to 2 mL of lysis buffer. Five microliters of GFP transcript (2200 gc/ $\mu$ L) were spiked into the lysed sample. After miniMAG or easyMAG extraction, the nucleic acid was eluted in 50 or 55  $\mu$ L of elution buffer, respectively.

## 2.3. Real-time RT-PCR

Real-time RT-PCR for the detection of CAL serogroup and CVV was performed using primers and probe developed in-house (CE-NC and CV-G1 primers and probes in Table 1). The CAL serogroup probe was labeled with the reporter 6-carboxyfluorescein (6-FAM) at the 5' end and a minor groove binder (MGB) (Applied Biosystems, Foster City, CA) at the 3' end. The CVV probe was labeled with the reporter VIC<sup>TM</sup> at the 5' end and an MGB (Applied Biosystems) at the 3' end. Amplification was carried out in a 25- $\mu$ L volume reaction using the One-Step Mastermix (Quanta Biosciences, Gaithersburg, MD), 0.5- $\mu$ L qScript One-Step Reverse Transcriptase (Quanta Biosciences), 450 nmol/L each of 4 CAL serogroup primers, 900 nmol/L each of 2 CVV primers, and 250 nmol/L each of CAL serogroup and CVV probes. 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 either ABI 7500 or 7900 instruments (Applied Biosystems).

## 3. Results

The sequences encoding the G1 glycoprotein of CVV and the N protein of CAL serogroup viruses from the GenBank database were aligned with the MEGA 4 software (Tamura et al., 2007). Regions that showed minimal sequence variation were chosen as candidate targets for primer/probe selection. Primers and probes were selected with the Primer Express software (Applied Biosystems) (Table 1). For detection of

the maximal number of CAL serogroup viruses, we selected 2 forward primers, 2 reverse primers, and 1 probe because we were unable to find 1 set of primers that were 100% homologous with all strains. The forward and reverse primers had 5 and 3 mismatches, respectively.

To perform quantification of genomic RNA for CAL serogroup and CVV, we prepared transcripts from 2 recombinant plasmids: 1 containing a 60-bp portion of the N ORF of LACV and the other a 301-bp portion of the G1 glycoprotein-encoding sequence of CVV (strain 62-7364).

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), qScript One-Step qRT-PCR kit (Quanta Biosciences), FailSafe Probes Real-Time PCR Optimization kit (EPICENTRE Biotechnologies, Madison, WI), SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen), QuantiTect Multiplex PCR kit (Qiagen, Valencia, CA), QuantiTect Probe RT-PCR kit (Qiagen), and LightCycler FastStart DNA Master Hybridization Probes kit (F. Hoffmann-La Roche, Nutley, NJ). The qScript One-Step qRT-PCR kit (Quanta Biosciences) presented a combination of the lowest Ct and highest  $\Delta$ Rn values (data not shown); therefore, we optimized the assay using this kit.

Primer optimization was performed with primer concentrations ranging from 300 to 1300 nmol/L, and probe optimization was performed with probe concentrations ranging from 50 to 300 nmol/L. For a TaqMan assay, optimal performance is achieved by selection of primer/probe concentrations that provide the lowest Ct and the highest  $\Delta$ Rn for a fixed amount of target template (Cirino et al., 2007). The Ct and  $\Delta$ Rn data taken together show that the optimal CAL serogroup virus primer concentrations were 450 nmol/L each, the optimal CVV primer concentrations were 900 nmol/L each, and the optimal probe concentrations for CAL serogroup and CVV were 250 nmol/L each.

Genomic RNAs from LACV and from CVV were quantified, and serial dilutions were made. Duplex real-time RT-PCR assay was performed on serial dilutions of the genomic RNAs. The assay detected a range from 75 to  $5.6 \times 10^6$  gc of LACV and a range from 30 to  $2.7 \times 10^7$  gc for CVV in a linear fashion.

Serial dilutions of LACV and CVV cultures were made and spiked into negative CSF specimens. After nucleic acid extraction and real-time RT-PCR, the limit of detection of the assay was determined multiple times. The sensitivity of the CAL serogroup assay was 100% at 75 gc of LACV target and 83% at 50 gc (performed 12 times). The sensitivity of the CVV assay was 100% at 30 gc of CVV target and 78% at 10 gc (performed 9 times). For quantified extracted genomic RNA, the limit of detection was 56 gc for the CAL serogroup assay, and 27 gc for the CVV assay (each performed 6 times).

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

Table 3  
CVV isolates used in this study

Sample name	Site of isolation (county in New York State)	Mosquito species	Collection date
40050036	Erie	<i>Aedes vexans</i>	2005
62-7364	NA	NA	1962
36010328	Saratoga	<i>Coquillettidia perturbans</i>	2001
37010185	Saratoga	<i>C. perturbans</i>	2001
35030381	Suffolk	<i>Ochlerotatus sollicitans</i>	2003
37030172	Dutchess	<i>Anopheles punctipennis</i>	2003
40030071	Orange	<i>Ochlerotatus trivittatus</i>	2003
39030254	Westchester	<i>O. trivittatus</i>	2003
39030270	Westchester	<i>Aedes cinereus</i>	2003
40040005	Orange	<i>A. vexans</i>	2004
40050212	Lewis	<i>C. perturbans</i>	2005
39050385	Monroe	<i>A. vexans</i>	2005

NA = not available.

organisms: Cox A9, Echo 9, HCoV 229E, hMPV, RSV, HRV, SARS coronavirus, adenovirus, CMV, EBV, HHV-6, HSV 1 and 2, VZV, WEE virus, HJV, DENV1–4, POW virus (representatives of lineage I and II), WNV, SLEV, *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 CAL serogroup and CVV 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 shown to be specific upon testing of a wide array of pathogens.

CAL serogroup viruses are related antigenically and genetically and are classified based on serologic assays. These assays together with sequence analysis have identified 3 distinct branches: CAL encephalitis, Melao, and Trivittatus complexes (Calisher, 1983; Campbell and Huang, 1999). To demonstrate that the real-time RT-PCR assay would detect the different members of this serogroup, we assessed detection of 11 different strains, representing all 3 complexes (Table 2). We performed the duplex real-time RT-PCR assay

on extracted RNA from each strain to determine whether nucleic acid from all 11 strains would be successfully amplified by the real-time RT-PCR reaction. Results showed that all strains were detected and amplified. In addition, nucleic acid was extracted from each of 12 isolates of amplified CVV (Table 3) and then tested in the duplex real-time RT-PCR assay. All isolates were detected and amplified.

The interassay precision of the real-time RT-PCR assay was determined by calculating coefficient of variation (CV) using data from 13 replicates of the assay positive controls. The CV was 3.9% for the CVV assay and 1.7% for the CAL serogroup assay. The intraassay precision was calculated by determining the variation among results obtained for 6 replicates of each positive control viral RNA. For the CVV assay, the CV was 1.4%, and for the CAL serogroup assay, it was 1.6%.

Because we were unable to obtain patient specimens that were positive for either CAL serogroup viruses or CVV, we spiked LACV and CVV cultures, at each of 3 dilutions, into negative CSF specimens. Appropriate dilutions of LACV culture were made to obtain high ( $5.6 \times 10^4$  gc/250- $\mu$ L CSF, equivalent to  $5.1 \times 10^3$  gc/reaction), medium ( $5.6 \times 10^3$  gc/250- $\mu$ L CSF, equivalent to  $5.1 \times 10^2$  gc/reaction), and low ( $5.6 \times 10^2$  gc/250- $\mu$ L CSF, equivalent to 51 gc/reaction) concentrations. Similarly, a range of virus culture concentrations that included high ( $6.5 \times 10^4$  gc/250- $\mu$ L CSF, equivalent to  $5.9 \times 10^3$  gc/reaction), medium ( $6.5 \times 10^3$  gc/250- $\mu$ L CSF, equivalent to  $5.9 \times 10^2$  gc/reaction), and low ( $6.5 \times 10^2$  gc/250- $\mu$ L CSF, equivalent to 59 gc/reaction) concentrations were selected for the CVV strain 62-7364. These dilutions of virus were then spiked into CSF that had previously tested negative for CAL serogroup viruses and CVV. Forty negative samples were either not spiked or else spiked with 1 of 3 concentrations of LACV and CVV in various combinations (Table 4). In addition to these samples, 1 negative extraction control was included in each extraction run. All 40 blinded samples and negative controls were also spiked with a known amount of GFP RNA transcript (2200

Table 4  
Mean Ct values obtained when the CAL serogroup, CVV, and GFP real-time RT-PCR assays were performed on extracts of CSF specimens spiked with LACV and CVV cultures

Sample	Virus spike	Mean Ct for viral assay	Ct range for viral assay	CV for viral assay	Mean Ct for GFP assay	Ct range for GFP assay	CV for GFP assay
NTC		$\geq 45$	$\geq 45$ to $\geq 45$	0	$\geq 45$	$\geq 45$ to $\geq 45$	0
<i>LACV spike</i>							
	Negative	$\geq 45$	$\geq 45$ to $\geq 45$	0	36.0	35.17 to 36.42	1.01%
	Low	35.55	34.75 to 39.0	3.98%	35.84	35.52 to 36.85	1.22%
	Medium	32.31	31.92 to 32.76	0.83%	36.04	35.34 to 36.61	1.18%
	High	28.29	27.85 to 29.08	1.27%	36.24	35.59 to 37.05	1.26%
<i>CVV spike</i>							
	Negative	$\geq 45$	$\geq 45$ to $\geq 45$	0	35.88	35.52 to 36.32	0.86%
	Low	36.16	34.19 to 40.64	5.31%	35.82	35.17 to 36.28	0.94%
	Medium	32.64	31.55 to 33.31	1.95%	36.1	35.34 to 36.6	1.09%
	High	29.59	29.1 to 30.38	1.58%	36.36	35.53 to 37.05	1.33%

The high, medium, and low spikes for LACV were  $5.1 \times 10^3$ ,  $5.1 \times 10^2$ , and 51 gc/reaction, respectively; the high, medium, and low spikes for CVV were  $5.9 \times 10^3$ ,  $5.9 \times 10^2$ , and 59 gc/reaction, respectively. The mean numbers are from 10 assays, except for the medium spike for CVV, which was from 9 assays. NTC = no template control.

gc/ $\mu$ L), 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).

There was minimal loss in sensitivity after nucleic acid extraction, as evidenced by detection of all blinded high, medium, and low gene copy spikes in CSF samples (Table 4). The negative extraction controls had Ct values of  $>45$ , indicating that cross-contamination had not occurred during extraction of any of the samples; consequently, no false positives were detected. In addition, all real-time RT-PCR experiments included positive LACV (300 gc/reaction) and CVV (1000 gc/reaction) controls, which gave Ct values within the respective acceptable ranges (31–34 and 29–32). The Ct values for GFP for all of the samples were also within the acceptable range (35–38). In general, if the GFP Ct value of a clinical specimen exceeds the acceptable range, the recommendation would be to repeat the extraction and real-time RT-PCR.

#### 4. Discussion

Human infections by CAL serogroup viruses and CVV are relatively rare. Nevertheless, they constitute an important public health concern because they are associated with severe morbidity and mortality. LACV, in particular, is a significant cause of pediatric encephalitis in the Midwestern United States (Griot et al., 1994). Disease due to CAL serogroup viruses is nationally reportable to the CDC.

Bunyavirus infections occur largely in areas with mosquito activity, during the summer and fall, when mosquitoes are feeding. In terms of geographic location, most cases of LACV infection occur in the Midwestern, mid-Atlantic, and Southeastern states. In the United States, approximately 70 cases are reported each year with a case fatality rate of  $<1\%$  (<http://www.cdc.gov/ncidod/dvbid/arbor/lacfact.htm>), although the disease is thought to be significantly underreported. JCV is distributed throughout much of North America, but infections have rarely been reported. The most common CAL serogroup viruses in Eurasia are TAHV and INKV (Demikhov et al., 1991). SSHV also circulates in northern Russia, and cases of Snowshoe hare encephalitis have been reported in North America (Artsob, 1983; Vanlandingham et al., 2002). CVV is endemic in Canada, Mexico, and the United States. Disease in humans caused by CVV is relatively rare, although cases have been reported in the United States (Campbell et al., 2006; Sexton et al., 1997).

Prevention is the key to reducing cases of bunyavirus infections. Mosquito control measures and avoidance of areas where the disease is endemic will help in reducing numbers of infected individuals. The surveillance of virologic activity in natural hosts, as well as surveillance of disease in humans, is

essential for early detection of outbreaks and for implementation of vector control measures.

Serology is the gold standard for diagnostic testing of arboviruses. Plaque reduction neutralization tests (PRNTs), hemagglutination inhibition (HI) tests, and immunoglobulin M (IgM) antibody-capture enzyme-linked immunosorbent (MAC-ELISA) assays have been used in the diagnosis of bunyaviruses (Calisher et al., 1986b; Grimstad et al., 1986; McJunkin et al., 1998). The disadvantages of these tests are that PRNT is time consuming and cumbersome, HI is not sensitive, and MAC-ELISA may not be sufficiently specific to determine the infecting serotype (Calisher, 1994; Calisher et al., 1986b). In addition, IgM antibodies appear to persist. For example, serum IgM antibodies to LACV remain elevated  $>9$  months after infection; therefore, detection of IgM in a single serum sample from an individual who lives in an endemic area is not indicative of recent infection (Calisher et al., 1986b; Tsai, 1991). Virus isolation from CSF and brain tissue is difficult and time consuming and is, therefore, not routinely performed on specimens from patients diagnosed with viral encephalitis (Calisher, 1994; Romero and Newland, 2006).

Molecular detection by real-time RT-PCR is an ideal method for detection of CAL serogroup viruses and CVV in humans and other vertebrate hosts because it is rapid, sensitive, specific, reproducible, and amenable to automation. Various RT-PCR methods, the majority of which use conventional RT-PCR methodology, have been employed for the detection of these viruses (Campbell and Huang, 1995; Chandler et al., 1998; Grady and Campbell, 1989; Kuno et al., 1996; Huang et al., 2001; Lambert et al., 2005; Ngo et al., 2006; Wasieloski et al., 1994).

We report the development of a duplex TaqMan real-time RT-PCR assay for the detection of CAL serogroup viruses and CVV. The targets for the PCR assay are sited within conserved regions of the genome, namely, sequences encoding the G1 glycoprotein of CVV and the N protein of CAL serogroup viruses. Although the assay is intended for patient CSF samples, it can also be used for vector surveillance. We found the optimal PCR kit for this assay to be the qScript One-Step qRT-PCR kit (Quanta Biosciences). The linear assay range is 75 to  $1.5 \times 10^6$  gc/reaction for CAL serogroup (specifically LACV) and 30 to  $2.7 \times 10^7$  gc/reaction for CVV. The sensitivity of the assay is 75 gc/reaction for LACV and 30 gc/reaction for CVV. 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 11 different viruses representative of the 3 complexes of CAL serogroup viruses and also the 12 different isolates of CVV that we tested (Tables 2 and 3).

There is little loss of virus or internal control during the nucleic acid extraction process, as evidenced by the spiking experiments in patient CSF specimens (Table 4). The assay correctly identified the CAL serogroup or CVV genome present in the specimen, regardless of the concentration of virus that was spiked. Moreover, although many of the



spiked samples had both viruses present, in some cases at high concentrations, 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. Furthermore, both detection assays, as well as the internal control assay, showed minimal variability, as demonstrated by the low CV values when the assay was performed multiple times (Table 4).

The use of the GFP internal control establishes an extraction and PCR inhibition control for the CAL serogroup and CVV detection assay that will allow inefficiencies in sample preparation and RT-PCR to be identified. The assay has a turnaround time of less than 5 h (including the nucleic acid extraction step) and is, therefore, rapid. Our data show that it is a highly specific, reproducible, and sensitive assay for detecting CAL serogroup virus RNA and CVV RNA in patient samples, and the multiplexed nature of the assay makes it cost-effective and especially suitable when sample volume is limited. In addition, the assay can be used in high-throughput screening for surveillance studies.

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