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Development of a Multi-Target TaqMan Assay to Detect Eastern Equine Encephalitis Virus Variants in Mosquitoes

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Abstract

Disease outbreaks caused by eastern equine encephalitis virus (EEEV; Togaviridae, *Alphavirus*) may be prevented by implementing effective surveillance and intervention strategies directed against the mosquito vector. Methods for EEEV detection in mosquitoes include a real-time reverse transcriptase PCR technique (TaqMan assay), but we report its failure to detect variants isolated in Connecticut in 2011, due to a single base-pair mismatch in the probe-binding site. To improve the molecular detection of EEEV, we developed a multi-target TaqMan assay by adding a second primer/probe set to provide redundant targets for EEEV detection. The multi-target TaqMan assay had similar performance characteristics to the conventional assay, but also detected newly-evolving strains of EEEV. The approach described here increases the reliability of the TaqMan assay by creating back-up targets for virus detection without sacrificing sensitivity or specificity.

Key Words: Eastern equine encephalitis virus—Real-time PCR—Surveillance—TaqMan.

Introduction

Surveillance for eastern equine encephalitis virus (EEEV; Togaviridae, *Alphavirus*) infection in field-collected mosquitoes is used to monitor virus amplification within enzootic cycles, provide advance warning of disease outbreaks, and guide vector control and other disease prevention efforts (Eldridge 2004). This is accomplished by collecting mosquitoes from endemic sites, sorting them into pools by species, and testing pooled specimens by cell culture and/or molecular assays (Andreadis et al. 1998). Real-time reverse transcriptase PCR techniques such as the TaqMan assay have been adopted for arbovirus surveillance because these methods are sensitive, provide rapid results, and do not require strict maintenance of a cold-chain and high levels of biocontainment in order to amplify viruses in cell culture (Lanciotti et al. 2000; Callahan et al. 2001; Lambert et al. 2003). Despite these advantages, TaqMan assays are designed to narrowly target only one or two pathogens of interest, thereby missing detection of new or unanticipated viruses in the environment, in contrast to culture-based methods (Armstrong et al. 2011). Furthermore, PCR methods may be less sensitive or may completely fail to detect mutated strains of their targets (Metzgar 2011), which is particularly problematic for RNA viruses such as EEEV that mutate and evolve more rapidly than DNA-based organisms (Jenkins et al. 2002; Arrigo et al. 2010). Broad-spectrum culture methods may be used in combination with virus-specific molecular methods to screen and then identify EEEV and other arboviruses from field specimens (Armstrong and Andreadis 2010), but this is not always feasible at the operational level. Therefore, molecular methods used for EEEV surveillance need to be sufficiently robust to detect the diversity of viral variants encountered in nature.

In 2011, we isolated four strains of EEEV in cell culture from mosquitoes collected during the statewide arbovirus surveillance program in Connecticut. All of these isolates tested negative by a conventional TaqMan assay for EEEV (Lambert et al. 2003), and were sequenced to search for mutations in primer- and probe-binding sites. An alternative, multi-target TaqMan assay was then developed to improve assay coverage that includes detection of newly-evolving EEEV strains.

Materials and Methods

Sample preparation and virus culture

Mosquito pools were processed for virus isolation by placing ≤50 mosquitoes in a 2-mL microcentrifuge tube with a copper BB and adding 1 mL of PBS-G (phosphate-buffered saline, 30% heat-inactivated rabbit serum, 0.5% gelatin, and 1× antibiotic/antimycotic). Mosquitoes were homogenized

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using a Vibration Mill MM300 (Retsch Laboratory, Irvine, CA) at 25 cycles per second for 4 min. The mosquito pools were centrifuged for 10 min at 520 g and supernatants (100 μL) were inoculated onto a monolayer of Vero cells growing in 25-cm^2 flasks. The cells were maintained at 37°C and 5% CO_2 and monitored for cytopathic effect from days 3–7 following inoculation. Virus cultures were harvested and stored at −80°C until further testing. Other viruses used in this study were obtained from our reference collection and had been previously passaged in suckling mice, BHK cells, and/or Vero cells.

RNA extraction and TaqMan assay

Viral RNA was extracted from 70 μL of mosquito pool homogenates or virus cultures using the QIAamp viral RNA kit (Qiagen, Valencia, CA), and eluted in an equal volume of elution buffer. TaqMan assays were performed using the TaqMan One-Step RT-PCR kit (Applied Biosystems, Foster City, CA), based on the manufacturer's specifications. For each reaction, 2.5 μL of extracted RNA was added to a master mix containing 1×PE buffer, 25 pmol of each primer, 3.75 pmol of probe, and 0.5 μL enzyme mix, for a final volume of 25 μL. Each primer/probe set listed in Table 1 was added either separately in single assays, or were combined in a single reaction for multi-target assays. The probes were labeled with FAM reporter dye at the 5′ end and TAMARA quencher dye at the 3′ end. Amplification was performed using the Smart Cycler System (Cepheid, Sunnyvale, CA) as follows: 50°C for 20 min, and 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. Cycle threshold (Ct) values were determined using automated threshold analysis software for the Smart Cycler System.

Nucleotide sequencing

Four EEEV isolates obtained in Connecticut in 2011 were partially sequenced to determine if there were mutations in the binding sites for primers/probe: 9391/9459c/9414probe (Table 1). A fragment of the E2 gene (710 bp) was amplified using primer pairs E2 fwd (TCCACAGTGCCAAGGTG AAAA) and E2 rev (TCGTCGGCTTAATGCAGCA), and the Titan One-Tube RT-PCR system (Roche Diagnostics, Indianapolis, IN), following the manufacturer's protocol. Amplification was performed as follows: 50°C for 30 min, 94°C for 2 min, 10 cycles of 94°C for 15 sec, 55°C for 30 sec, and 68°C for 45 sec, followed by 25 cycles of 94°C for 15 sec, 55°C for 30 sec, and 68°C for 45 sec + 5 sec per cycle, and 1 cycle of 68°C for 7 min. Amplification products were purified using the QIAquick PCR purification kit (Qiagen), and sequenced using primers E2 fwd and E2 rev at the Keck Sequencing Facility (New Haven, CT).

Plaque titration

Plaque titrations were performed on Vero cells growing in 12-well plates. Ten-fold virus dilutions were inoculated onto Vero cell monolayers and absorbed for 1 hour with periodic rocking. Cell cultures were overlaid with 1% methyl cellulose in minimal essential medium and 5% fetal bovine serum, and then fixed and stained with 1% crystal violet after a 3-day incubation period.

Results

EEEV was isolated in cell culture from four mosquito pools collected in Connecticut during 2011, comprising three pools of *Culiseta melanura* (Coquillett), and one pool of *Ochlerotatus canadensis* (=*Aedes canadensis*) (Theobald). All four virus isolates were positive for EEEV by TaqMan assay when using the in-house capsid primers 411F/527R/463 probe (mean Ct = 13.7–15.4), but were consistently negative when the published E2 primers/probe 9391/9459c/9414probe (Ct = > 50) were used (Lambert et al. 2003), during three separate replications. EEEV isolates were then partially sequenced to characterize primer and probe binding sites. Virus sequences were identical to each other and matched to primers 9391and 9459c, but differed from the 9414 probe sequence by a C to T substitution at genome position 9428. These results indicate that a single-nucleotide substitution within the probe binding site may lead to TaqMan assay failure. This is the first time that we have encountered false-negative results using the published TaqMan assay since its adoption into the statewide surveillance program in 2003.

To increase the reliability of EEEV detection by TaqMan assay, we developed a multi-target assay that combines both primer/probe sets (411F/527R/463probe and 9393/9459c/9424probe) listed in Table 1 to provide redundant targets for EEEV detection. The sensitivity of the multi-target assay was evaluated by comparing it to the previously-published single-target assay using primers/probe 9391/9459c/9414probe alone (Lambert et al. 2003). Ten-fold dilutions of viral RNA from previously titrated virus culture were added to each assay in duplicate. Both assays yielded similar Ct values and both reliably detected EEEV down to 0.32 PFU (mean Ct = 36.8 and 37.4, for the single-target and multi-target assays, respectively) in parallel comparisons (Table 2). Test results were considered equivocal at the 0.032 PFU dilution (mean Ct = 45.4 and 46.1, respectively), and negative (Ct < 50) at lower

Table 1. Primers and Probes for Eastern Equine Encephalitis Virus (EEEV) Detection by the TaqMan Assay

Primer name	*Sequence (5′-3′)*	*Genomic position*[a]	*Product size (bp)*	*Reference*
411F	GAACGGACAGGTGAATGGTT	Capsid 7948–7967	117	This article
527R	CTGGCCTTCTTCAGCTTGAT	Capsid 8064–8045		
463probe[b]	CCGCTGCACGTAGAAGGCAGA	Capsid 8000–8020		
9391	ACACCGCACCCTGATTTTACA	E2 9394–9414	69	Lambert et al. 2003
9459c	CTTCCAAGTGACCTGGTCGTC	E2 9462–9442		
9414probe[b]	TGCACCCGGACCATCCGACCT	E2 9417–9437		

[a]Based on the sequence of EEEV strain Florida 91-4697 (Genbank accession no. AY705241).
[b]Labeled with FAM (5′ end) and TAMARA (3′ end).

Table 2. Comparison of for Eastern Equine Encephalitis Virus (EEEV) Detection by Single- and Multi-Target TaqMan Assays

Virus dilution[a]	*PFU/assay*	*Mean Ct*	
		Single-target	*Multi-target*
10^{-3}	318	24.9	23.4
10^{-4}	32	28.5	27.1
10^{-5}	3.2	31.9	31.4
10^{-6}	0.32	36.8	37.4
10^{-7}	0.032	45.4	46.1
10^{-8}	0.0032	>50	>50
10^{-9}	0.00032	>50	>50

[a]EEEV strain 10911-09 (Connecticut 2009).

dilutions. The addition of a second primer/probe set did not significantly alter the sensitivity of EEEV detection by the TaqMan assay.

To determine whether the multi-target TaqMan assay is specific for North American strains of EEEV, we tested the assay against a panel of EEEV cultures from different geographic regions and years, in addition to three related alphaviruses: Highlands J virus (HJV), western equine encephalitis virus, and Venezuelan equine encephalitis virus; and two sympatric North American arboviruses: West Nile virus (WNV) and Jamestown Canyon virus (JCV; Table 3). The multi-target assay detected all of the North American isolates of EEEV on the panel, including one of the aforementioned EEEV strains from Connecticut in 2011, but did not detect the South American strains of EEEV, or any of the other arboviruses tested. Nucleotide distances between the North and South American varieties of EEEV are greater than 20%, and they have been proposed as separate species within the EEE complex (Arrigo et al. 2010).

To evaluate its utility in field-collected mosquitoes, we extracted RNA from mosquito pools that had been previously tested in Vero cell culture and tested them using the multi-target assay. The panel comprised 7 mosquito species of the genera *Aedes, Coquillettidia, Culex, Culiseta*, and *Ochlerotatus* from Connecticut that were either negative or infected by EEEV, HJV, JCV, or WNV by cell culture assay (Table 4). The multi-target TaqMan assay detected EEEV in the four mosquito pools positive by cell culture, but not in any of the remaining mosquito pools. These results confirm that the assay is specific for its intended target and may be used to detect EEEV in field-collected mosquitoes.

Discussion

A number of PCR-based methods have been adopted for use in arbovirus surveillance programs to either supplement or replace the diagnostic capabilities of traditional culture-based methods (Shi and Kramer 2003; Powers and Roehrig 2011). Real-time reverse transcriptase PCR systems such as the TaqMan assay are often used as a primary screening tool because they provide results within hours and permit quantification of viral RNA based on Ct values. These assays use a closed-tube system that minimizes the risk of amplicon carryover, and are adaptable to the high-throughput screening formats required for surveillance programs (Shi et al. 2001). Nevertheless, despite these strengths, TaqMan assays are limited to detecting only the viruses being tested, and may fail to detect their intended targets due to viral mutations as demonstrated in this study. We could not detect any of the EEEV strains isolated in 2011 by a conventional TaqMan assay due to a single base pair mutation within the probe target sequence. This is consistent with the findings of a previous study by Papin and associates 2004, in which they evaluated the performance of a WNV TaqMan assay against a panel of synthetic oligonucleotide targets containing point mutations within the probe binding site. The assay failed to detect 47% of the possible single-nucleotide variations and all targets containing more than two nucleotide mutations. False-negative results due to strain variability have also been documented for herpes simplex and avian influenza viruses, raising further concerns about the efficacy of these methods (Anderson et al. 2003; Xing et al. 2008).

Cell culture assays are inherently sensitive for detecting a wide variety of arboviruses and virus strains. These methods are based on the ability of viruses to infect, replicate, and cause cytopathic effect in a particular cell line, which is a highly conserved phenotype of pathogenic viruses. Specific molecular methods, in contrast, are more prone to assay failure when detecting novel or mutant virus strains. Rapidly

Table 3. Specificity of the Multi-Target TaqMan Assay for North American Strains of Eastern Equine Encephalitis Virus (EEEV)

Virus	*Strain no.*	*Geographic origin and year isolated*	*TaqMan Ct*
EEE	Ten Broeck	Virginia, USA 1933	16.1
EEE	82V2137	Florida, USA 1982	12.5
EEE	CT36	Connecticut, USA 1996	16.5
EEE	9744-01	Connecticut, USA 2001	13.6
EEE	NY435	New York, USA 2004	12.0
EEE	MA415	Massachusetts, USA 2007	12.4
EEE	18410-11	Connecticut, USA 2011	13.8
EEE	GM90386	Panama, 1984	>50
EEE	BeAr300851	Brazil, 1975	>50
Highlands J	3591-98	Connecticut, USA 1998	>50
Western equine encephalitis	McMillan	Canada, 1941	>50
Venezuelan equine encephalitis	TC-83	Trinidad, 1943	>50
West Nile	3667-10	Connecticut, USA 2010	>50
Jamestown Canyon	2358-10	Connecticut, USA 2010	>50

Table 4. Detection of Eastern Equine Encephalitis Virus (EEEV) in Mosquito Pools by Vero Cell Culture or Multi-Target TaqMan Assay

Pool no.	*Mosquito species*	*No. of mosquitoes*	*Vero cell culture*	*TaqMan Ct*
1	*Culiseta morsitans*	1	Neg	>50
2	*Coquillettidia perturbans*	2	Neg	>50
3	*Culiseta melanura*	1	HJV+	>50
4	*Culiseta melanura*	2	EEEV+	26.3
5	*Aedes vexans*	36	Neg	>50
6	*Ochlerotatus canadensis*	50	EEEV+	21.1
7	*Culiseta melanura*	50	HJV+	>50
8	*Culiseta melanura*	5	Neg	>50
9	*Culex restuans*	2	HJV+	>50
10	*Culex pipiens*	25	WNV+	>50
11	*Culiseta melanura*	20	EEEV+	22.6
12	*Culiseta melanura*	50	EEEV+	20.4
13	*Ochlerotatus canadensis*	3	Neg	>50

evolving viruses may acquire synonymous or silent nucleotide mutations within primer and probe target sequences, allowing them to evade detection at little or no adaptive cost to the virus (Metzgar 2011). One solution is to develop diagnostic assays targeting highly conserved regions of the virus genome. This is an important consideration, but balancing the requirements for primer and probe design (i.e., amplicon size, melting temperature, GC content, primer dimerization, and secondary structure) place constraints on the selection of gene targets for assay development; moreover, it is not always possible to predict which regions will remain conserved from limited sequence comparisons. Based on these considerations, we and others (Rondini et al. 2008; Papin et al. 2010) support the use of multiple, parallel tests to prevent single-nucleotide mutations from blocking virus detection. The multi-target TaqMan assay developed in this study provides proof of this principle by using two redundant targets for EEEV detection.

TaqMan assays may be combined to detect multiple targets of the same pathogen as shown in this study, or to detect multiple pathogens in multiplex reactions (Dyer et al. 2007). This flexibility allows one to rapidly extend assay coverage when a new virus or virus variant emerges in an area. We accomplished this by adding our in-house primer/probe set to a previously published TaqMan assay with no further modification. The resulting assay was equally sensitive to the single-target assay, specific for North American strains of EEEV, and could be used in mosquito pools. Our multi-target assay provides an expedient solution to account for newly-emergent variants of EEEV, and this approach could be applied to the molecular diagnostics of other rapidly-evolving viruses. In the meantime, it will be important to monitor the continued efficacy of molecular assays for their viral targets, and consider combining assays when appropriate. If mutations arise too frequently or become fixed within target regions, then primer and probes will need to be completely redesigned to ensure adequate coverage for contemporaneous virus strains.

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Author Disclosure Statement

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